

**ESSENTIAL FATTY ACIDS, GASTRO-DUODENAL PROSTAGLANDIN
METABOLISM, AND GASTRIC MUCOSAL PROTECTION**

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SUMMARY

This thesis examines the hypothesis that dietary essential fatty acids influence gastric prostaglandin metabolism and acid secretion and could modify the natural history of duodenal ulcer.

In healthy controls dietary supplementation with linoleic acid resulted in an increase in the mean output of PGE and its main metabolite, a reduction in mean serum gastrin concentration, a fall in acid secretion and an increase in the output of soluble mucus. In a group of patients with healed chronic duodenal ulcers the mean output of PGE was significantly less than that found in the controls, whilst the mean output of the PGE metabolite was higher. Furthermore, dietary linoleic acid did not increase prostaglandin output, nor did it affect acid secretion. These findings showed abnormal prostaglandin metabolism in duodenal ulcer, implying increased PGE catabolism and a failure to respond normally to dietary essential fatty acid.

Gastric mucosal injury was induced in normal subjects using aspirin and 80% ethanol. Linoleic acid pre-treatment had no beneficial effect upon mucosal injury in either experimental model, questioning the role of endogenous prostaglandins in mucosal protection.

The adipose fatty acid profile of duodenal ulcer patients

and matched controls was measured since this reflects chronic dietary fatty acid intake. Mean adipose % linoleic acid was significantly decreased in the ulcer group suggesting that dietary essential fatty acids may be important in peptic ulcer.

DECLARATION

The work in this study is original and was performed by myself. Work performed by others has been acknowledged in the text. Some of the work has already been published.

Grant HW, Palmer KR, Kelly RW, Wilson NH, Misiewicz JJ (1988) Dietary linoleic acid, gastric acid and prostaglandin secretion. Gastroenterology 94:955-959.

Permission has been granted for publication of this paper in this thesis.

HUGH W. GRANT

ETHICAL PERMISSION

Ethical permission was obtained from the Lothian Ethical Committee for Medicine and Oncology for each study, informed consent was obtained from each volunteer and patient.

STATISTICAL METHODS

Wilcoxon's sign rank method for paired data was used throughout this study. Each person acted as his own control or compared with an age-matched control. Student's "t" test was used for the analysis of data which was normally distributed. Results have been expressed as means with standard error of the mean (SEM) in brackets.

CHAPTER I

INTRODUCTION

1. **HYPOTHESIS AND AIMS**
2. **LINOLEIC ACID AND PROSTAGLANDIN METABOLISM**
 - Biosynthesis
 - Control of the prostanoid cascade
 - Degradation and metabolism
3. **PROSTAGLANDINS AND GASTRIC PHYSIOLOGY**
 - Acid secretion
 - Gastric mucosal protection
4. **DUODENAL ULCER AND PROSTAGLANDINS**

CHAPTER 1

1. HYPOTHESIS AND AIMS

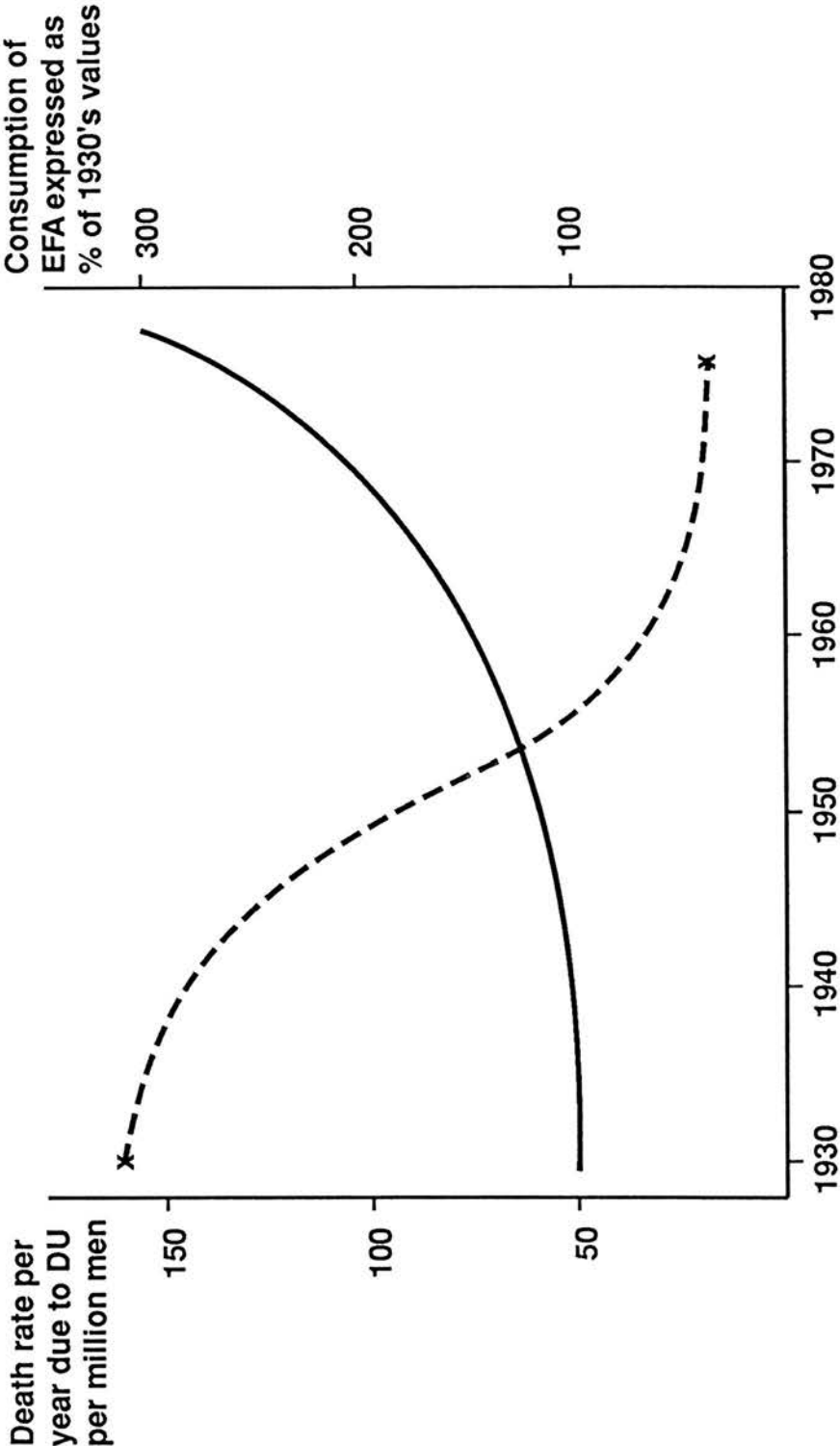
This thesis was stimulated by the hypothesis that the decline in the incidence and virulence of peptic ulcer disease may be related to the marked increase in the dietary availability of linoleic acid (Hollander and Tarnawski, 1986).

There has been a significant decline in the age-specific mortality from duodenal ulcer disease of men in their fifth decade in Scotland from 154 deaths/million men in the 1930's to 24 deaths/million men in 1976 (Sonnenberg et al, 1985) (Fig 1:1). During this same period a 200% increase in the consumption of linoleic acid and other essential fatty acids has been reported (HMSO 1982, Welsh and Marston, 1982).

These findings could be of aetiological importance in peptic ulcer disease because linoleic acid is a major substrate for prostaglandin synthesis in the gastro-duodenal mucosa. When linoleic acid is given orally or intragastrically the output of prostaglandins rises significantly (Grant et al, 1988 ; Hollander et al, 1982). Prostaglandins given orally can prevent mucosal ulceration induced experimentally by aspirin, alcohol, bile salts or boiling water (Robert et al, 1979 ; Miller, 1983). They also promote gastric mucosal repair

Fig 1:1

Decline in death rate due to duodenal ulcer
and increase in consumption of Essential
Fatty Acids (EFA)



following ulceration (Tarnawski et al, 1983).

Most of the evidence for the protective role of prostaglandins on the gastric mucosa comes from studies involving the oral administration of prostaglandins or their synthetic analogues. The stimulation of endogenous prostaglandin formation by dietary precursors (linoleic acid) provides an ideal opportunity to study the role of gastric endogenous prostaglandins.

The aims of this thesis were to determine in man whether,

- (1) Endogenous gastric prostaglandin formation could be stimulated by dietary linoleic acid.
- (2) To define the effects of dietary linoleic acid upon gastric acid output, fasting serum gastrin concentration and gastric mucus output.
- (3) To determine whether patients with duodenal ulcer disease have an abnormality of prostaglandin synthesis or degradation.
- (4) To determine whether endogenously synthesised prostaglandins protect against aspirin and alcohol-induced gastric mucosal injury.
- (5) To compare the dietary linoleic acid intake in duodenal ulcer patients with normal subjects.

2. LINOLEIC ACID AND PROSTAGLANDIN METABOLISM

Prostaglandins (PG's) constitute a family of chemically related fatty acids which are distributed throughout the body and modulate innumerable biological functions. Their existence was demonstrated over fifty years ago when Kurzok (1930) noted that strips of uterus relax or contract when exposed to semen. In the late 1950's Bergstrom and Sjovall (1960) isolated prostaglandins in a pure form and later defined their chemical structure.

Prostaglandins are the products of poly-unsaturated fatty acid metabolism of which the most important are linoleic acid and arachidonic acid. These are released from membrane-bound phospholipids by the action of phospholipase A₂ in response to a variety of physical, chemical and neuro-hormonal factors. Linoleic acid is an essential fatty acid with 18 carbons and 2 double bonds at the C9 and C12 positions (Fig 1:2). Arachidonic acid has 20 carbons and 4 double bonds and is rapidly metabolised to oxygenated products by the cyclo-oxygenase and lipoxygenase pathways. The intermediate cyclo-oxygenase products are converted to prostaglandins, the lipoxygenase products to leukotrienes (Fig 1:3).

The natural prostaglandins are analogues of prostanoic acid which has a cyclopentane ring and two side chains (Fig 1:2). There are several different classes - the suffices A, B, C, D, E, F denote different constituents of the ring and the numerical subscript - 1, 2, 3 denote

Fig. 1:2 Structures of Prostaglandins

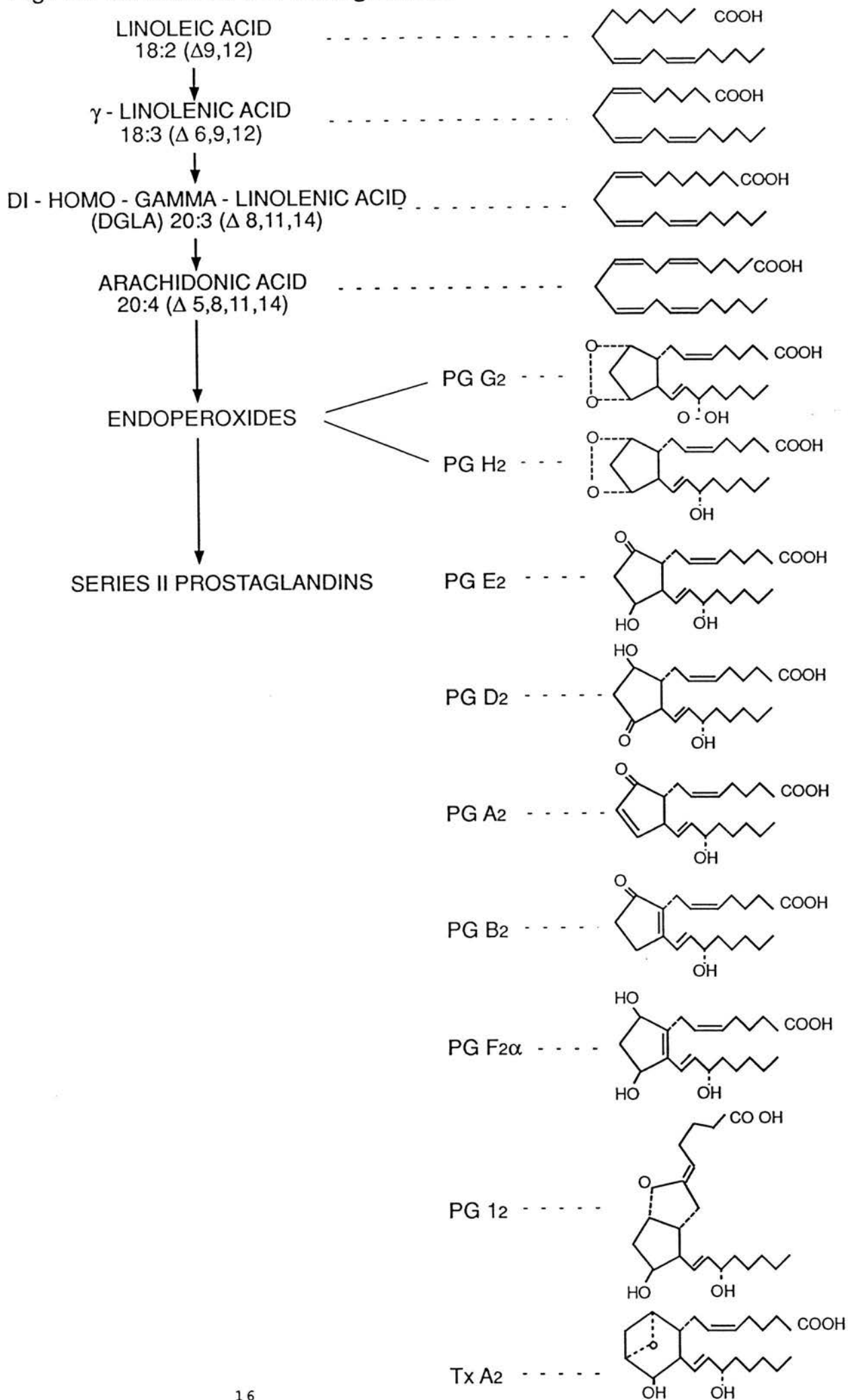
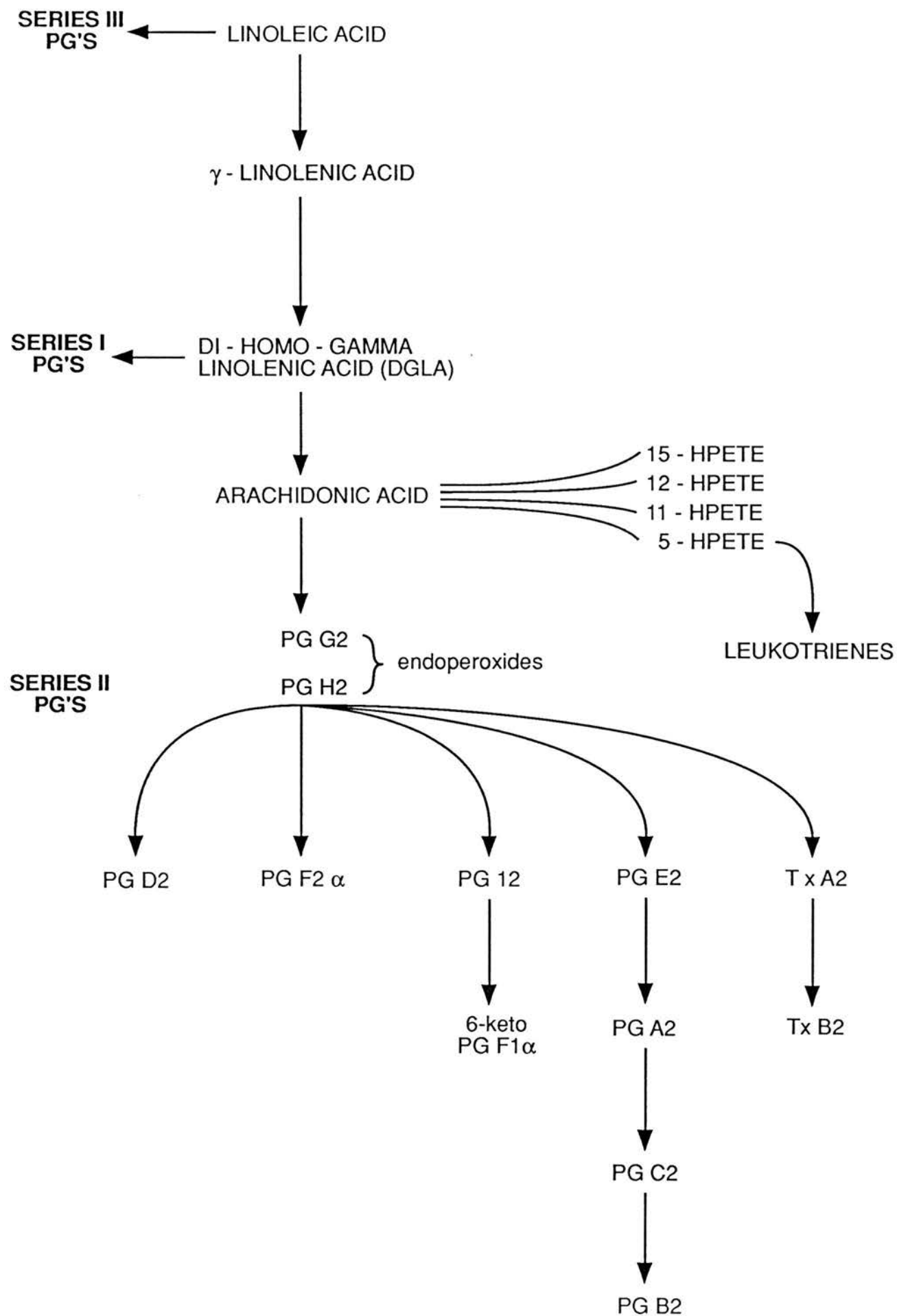


Fig. 1:3 Prostaglandin Pathway



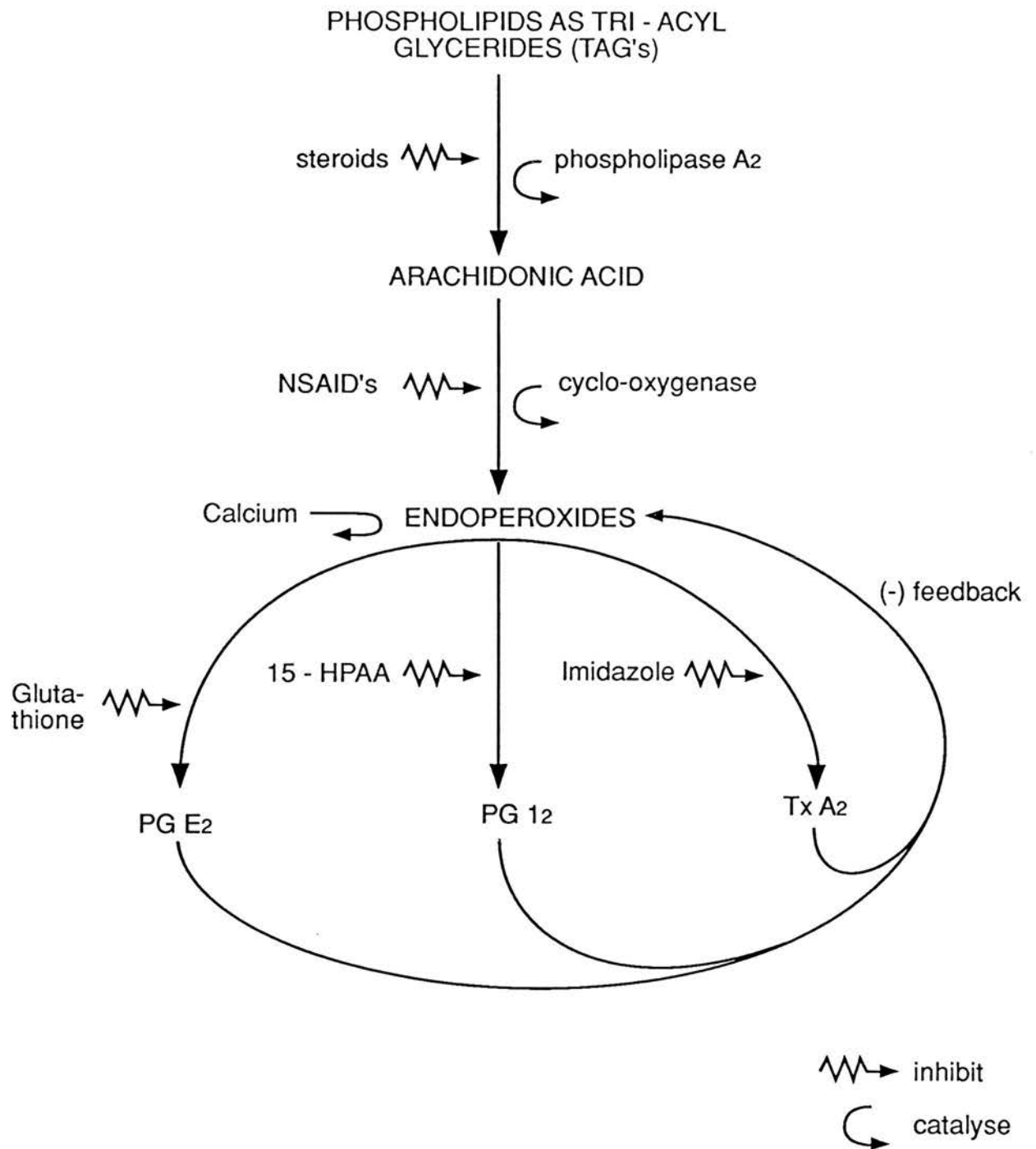
the number of double bonds on the side chain.

Biosynthesis

After linoleic acid is absorbed from the gastrointestinal tract it is converted into longer chain fatty acids in the liver eg. di-homo γ - linolenic acid, and then stored as a tri-acyl-glyceride (TAG). The formation of prostaglandin precursors from dietary linoleic acid and γ -linolenic acid depends on the nutritional status of the patient and the presence of other fatty acids which may compete for the cyclo-oxygenase and lipoxygenase enzyme complexes. Deficiency of essential fatty acids can result in low prostaglandin output whereas dietary supplementation can lead to increased output.

The common precursor of prostaglandins, thromboxanes (TX), and leukotrienes are three naturally occurring eicosapolyenoic acids (eicosa = 20); eicosatrienoic acid (or di-homo γ -linolenic acid), eicosatetraenoic acid (or arachidonic acid) and eicosapentaenoic acid. In man arachidonic acid is by far the commonest and gives rise to the series II prostaglandins. It is either derived from linoleic acid or ingested itself. It is esterified and stored as tri-acyl-glycerides throughout the body as a component of phospholipids in cell membranes. Hydrolysis of these esters provides the first rate controlling step of prostaglandin formation (fig 1:4).

Fig. 1:4 Regulation of Prostaglandin Metabolism



CO-FACTORS: IRON
HAEM
CYTOCHROMES
ASCORBIC ACID
COPPER

H₂O₂
ADRENALINE
ANGIOTENSIN II
ACETYL CHOLINE
5-HT

INHIBITORS: OTHER FATTY ACIDS
PRODUCT ANALOGUES
NSAID's

The specific metabolites of the arachidonic acid cascade vary between tissues and species. The first product of cyclo-oxygenase is an unstable cyclic endoperoxide PGG_2 which can proceed directly or via a peroxidase to PGH_2 , the common intermediate for TXA_2 , PGD_2 , PGE_2 , PGI_2 and $\text{PGF}_{2\alpha}$. An endoperoxide isomerase converts PGH_2 to either PGE_2 or its isomer PGD_2 . The combined action of this isomerase and reductase yields $\text{PGF}_{2\alpha}$. In some tissues 9-keto reductase catalyses the conversion of PGE_2 to $\text{PGF}_{2\alpha}$. PGE_2 may then undergo dehydration to PGA_2 and isomerisation to PGB_2 and PGC_2 . PGH_2 can be converted by the enzyme prostacyclin synthetase to PGI_2 which is very unstable, hydrolysing to its stable metabolite 6-keto $\text{PGF}_{1\alpha}$ (Granstrom, 1981). The other main route of PGH_2 metabolism is to TXA_2 . This is yet another unstable and highly active compound formed by the thromboxane synthetase enzyme complex. TXA_2 is hydrolysed non-enzymatically to the hemiacetal oxane TXB_2 .

The chain of events leading to prostaglandin and thromboxane synthesis can be initiated by diverse stimuli such as deformation of cell membranes, stretching of blood vessels or visceral distension (Piper and Vane, 1971 ; McGiff et al, 1972) ; even eating causes increased prostaglandin output (Ahlquist et al, 1983). Any damage to tissues (Granstrom et al, 1982) such as acute ischaemia, trauma, hypoxia and inflammation (Crampton et al, 1987; Williams, 1983) can lead to the release of lysosomal enzymes; these include

phospholipase A₂ which in turn hydrolyses linoleic and arachidonic acids from their membrane-bound phospholipids.

Not all metabolites are formed in all tissues and the relative amounts of cyclooxygenase and lipoxygenase vary between tissues. Most tissues seem to be able to synthesise prostaglandin endoperoxides from free arachidonic acid but the factors which control the subsequent steps are poorly defined. Lung, spleen, gastro-intestinal tract, thyroid and adrenals are able to synthesise a wide range of products whereas other tissues are more selective: mast cells produce mainly PGD₂, seminal vesicles mainly PGE₂, vessel walls mainly PGI₂ and platelets mainly TXA₂.

Control of the prostanoid cascade

Non-steroidal anti-inflammatory drugs (NSAIDs) are potent inhibitors of prostaglandin synthesis, they prevent the formation of endoperoxides. The sensitivity of cyclooxygenase to NSAID's varies between tissues (Ferreira, 1981). Aspirin inhibits the production of TXA₂ in platelets at doses which do not affect the production of PGI₂ in the endothelial cells, thereby reducing platelet aggregation.

Glucocorticoids reduce prostaglandin synthesis by inducing proteins (lipomodulins) which have anti-

phospholipase properties and so block their release from their biosynthetic site (Fig 1:4) (Hawkey et al, 1982).

Substrate analogues, end products and leukotrienes all have feedback on the pathway. There are also a large number of "permissive" hormones and co-factors, including ADH, angiotensin II, noradrenalin and bradykinin, which increase phospholipase A₂. TSH, insulin and ACTH stimulate prostaglandin production from arachidonic acid. Other co-factors include iron, cytochromes, ascorbic acid, acetyl choline and copper (Sih et al, 1970).

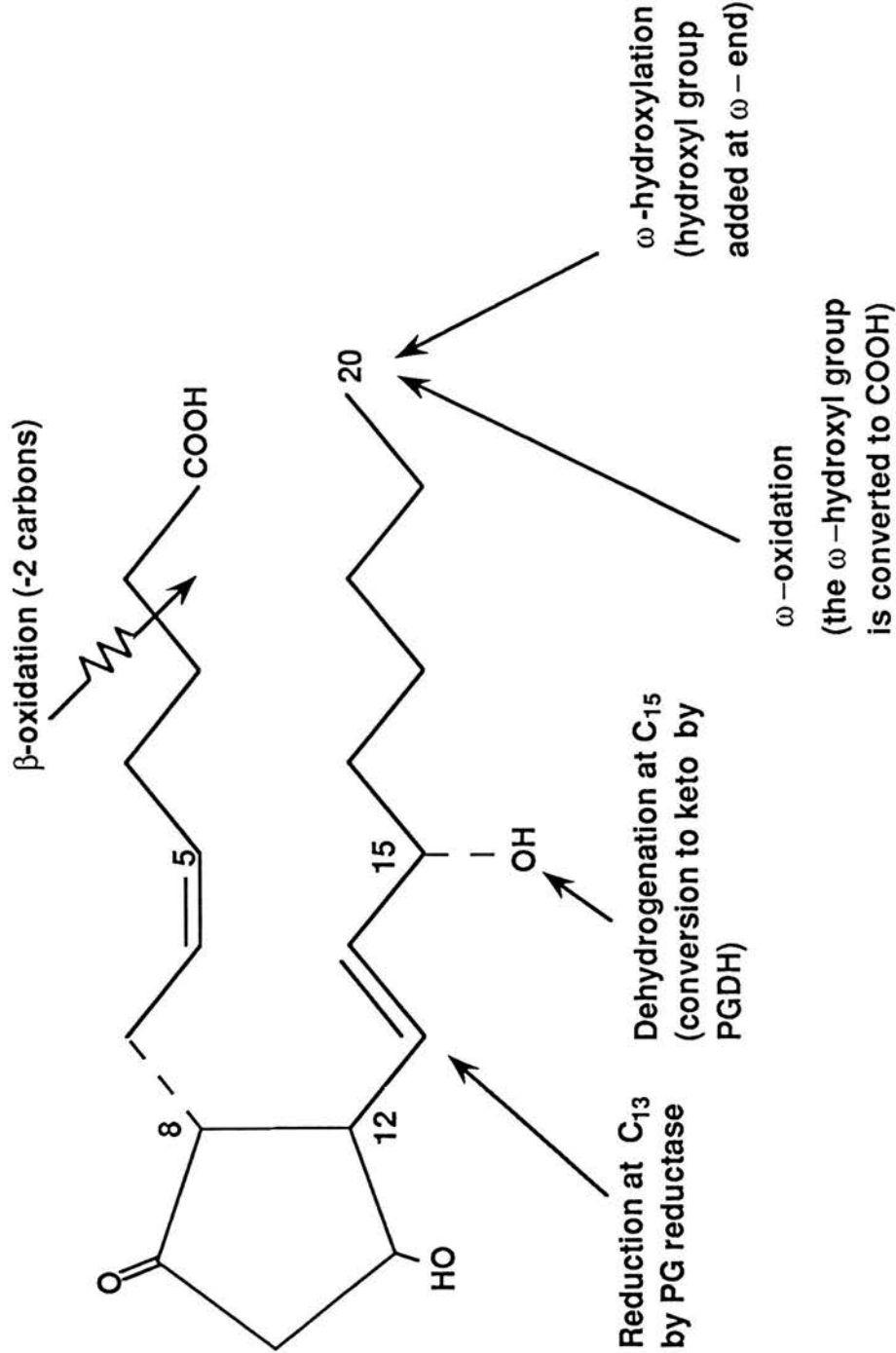
Prostaglandins are not stored but they are produced in response to the appropriate stimuli.

Degradation and metabolism

There are many mechanisms by which prostaglandins are catabolised or inactivated. The prostaglandin endoperoxides are highly unstable and only exist momentarily in vivo. Other prostaglandins have longer half-lives but still only survive minutes in the acid medium of the stomach. It can be difficult to measure their concentration in vivo so their more stable metabolites are measured as an indirect marker.

The first step in prostaglandin degradation is the oxidation of the C15 hydroxyl group by 15-hydroxy prostaglandin dehydrogenase (PGDH) (Fig 1:5). The 15-keto group is then reduced by Δ -3 reductase (PGR) to the

Fig 1:5 Prostaglandin E degradation and metabolism



13,14-dihydro Δ -3 derivatives. Subsequent β -oxidation and ω -oxidation of the side chains occurs giving rise to the dicarboxylic acids. Both PGDH and PGR are intracellular enzymes.

TXA₂ is very unstable and converts to TXB₂ spontaneously which then follows the main catabolic route. PGI₂ is also hydrolysed spontaneously to inactive 6-keto PGF_{1 α} .

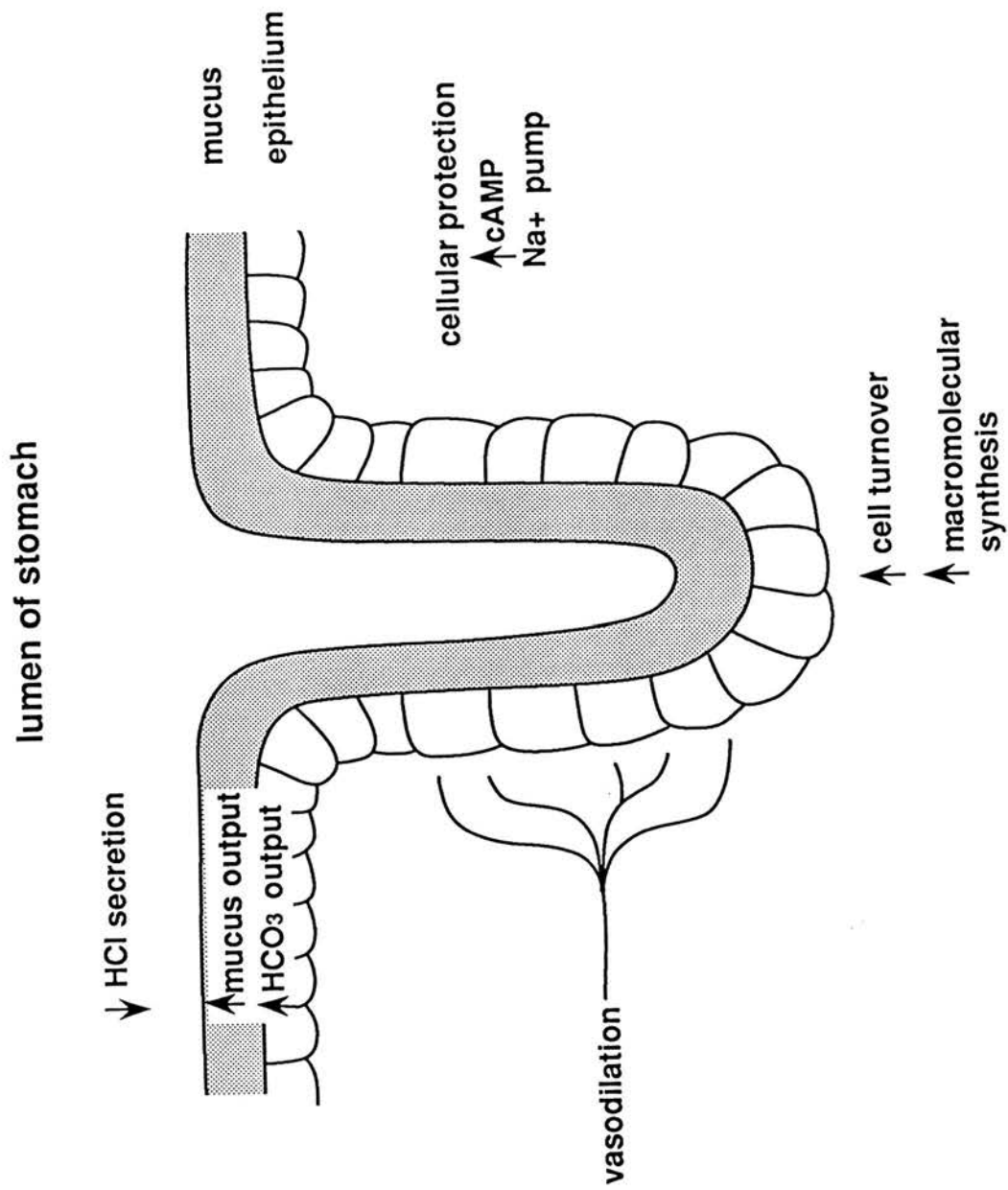
3. PROSTAGLANDINS AND GASTRIC PHYSIOLOGY

Prostaglandin E, F and I₂ are all synthesised throughout the gut. (Moncada et al, 1977; Ahlquist et al, 1982; Bennett et al, 1977; Peskar et al, 1980). In man the predominant prostaglandin in the gastro-intestinal tract is PGE (Bennett et al, 1968). This discussion will therefore concentrate on PGE and its main metabolite 13, 14-dihydro 15-keto PGE₂ (PGEM). The effects of PGE on the stomach and duodenum are summarised in Fig 1:6.

Acid secretion

One of the most important effects of prostaglandins on the stomach is acid inhibition (Johansson, 1985). This occurs whether the prostaglandin is given intravenously or orally (Classen et al, 1971; Horton et al, 1968). Pharmacological analogues are also potent acid inhibitors when given intravenously or orally (Konturek et al, 1976)

Fig 1:6 Modes of action of prostaglandin E on the gastric mucosa



The mechanisms by which prostaglandins act on the cell are not fully understood. In dogs prostaglandins have a greater effect on the stomach when administered topically onto the gastric mucosa rather than into the duodenum (after surgical ligation of the pylorus) suggesting a direct effect on the cell (Konturek et al, 1978; Robert, 1981). In human experiments PGE₂ analogues are also more effective when given intragastrically than into the duodenum or jejunum (Nylander and Andersson, 1974).

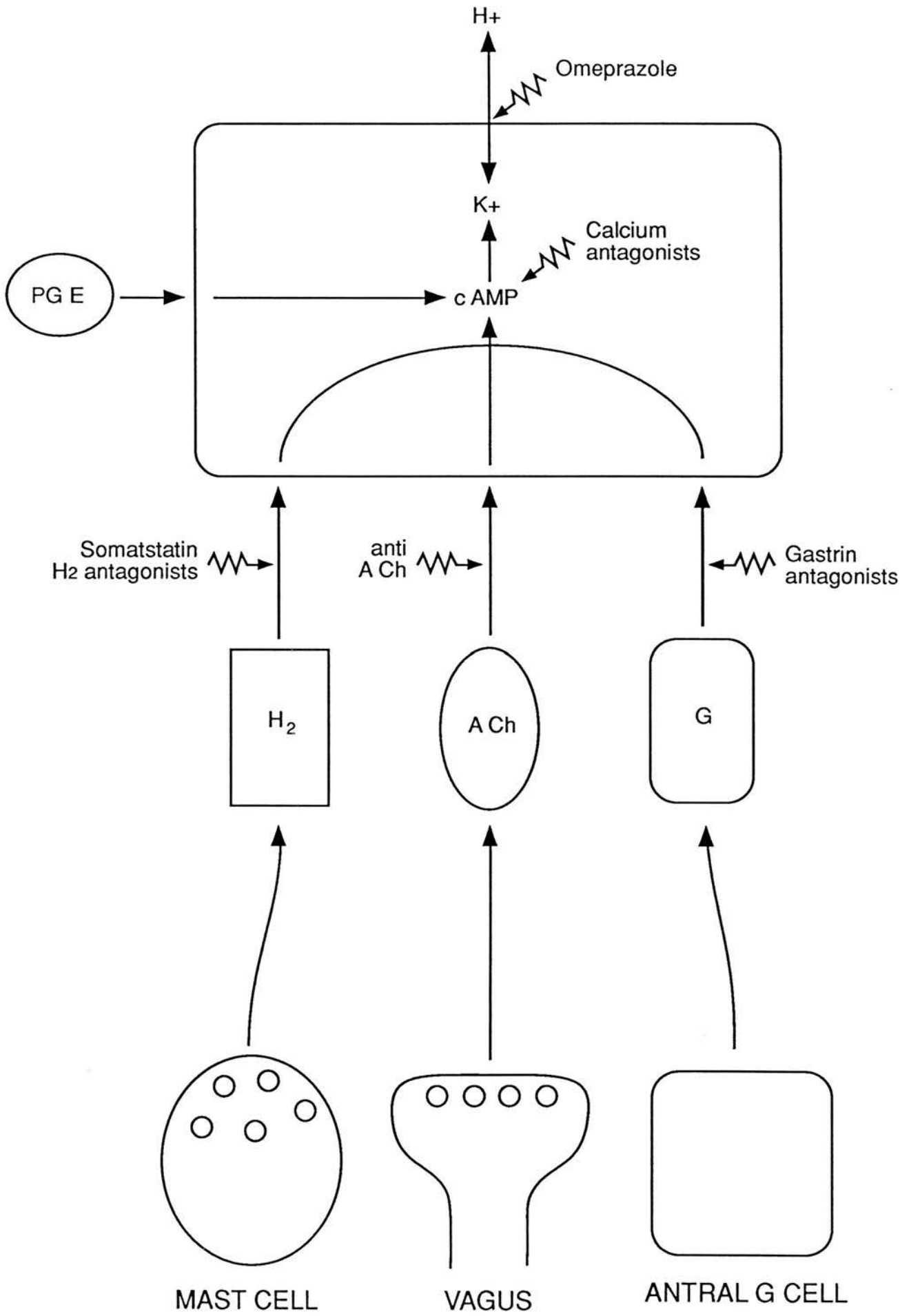
At a cellular level it is likely that prostaglandins inhibit acid secretion at a step beyond the H₂ receptor locus and reduce intracellular cAMP formation (Fig 1:7). They thus block the main pathway for the excitation of parietal cells by all secretagogues (Levine et al, 1982; Soll, 1980).

Prostaglandins may also mediate gastrin. In normal subjects PGE analogues suppress the gastrin response to a meal (Ippoliti et al, 1981). The effect of prostaglandin on gastrin in patients with duodenal ulcer is controversial (Mahachai et al, 1985; Tytgat et al, 1981), and will be discussed in chapter 2.

Gastric mucosal protection

The other effects of prostaglandins on the gastric mucosa can be broadly grouped under the heading "cytoprotection" i.e. the ability to protect the gastric mucosa against

Fig.1:7 Action of prostaglandin E on the parietal cell



injury independent of an acid-reducing effect. Robert (1984) defines cytoprotection as "a label used to describe the package of physiological mechanisms which protect gastric and intestinal mucosa from acid, pepsin, bile and other known ulcerogens". Cytoprotection is a poorly understood concept and involves a number of mechanisms. It seems to be a non-specific effect because almost every prostaglandin confers some protection although not all prostaglandins suppress acid secretion (eg. $\text{PGF}_{2\alpha}$).

Exogenous prostaglandins given in doses insufficient to suppress acid secretion can prevent gross mucosal lesions in the stomach against a wide variety of irritants (Robert et al, 1979). Small doses of gastric irritants can protect against subsequent larger doses by stimulating endogenous prostaglandins; this phenomenon was labelled "adaptive cytoprotection" by Robert in 1979. It has been further studied by Konturek et al (1982). This stimulation of endogenous prostaglandins has been implicated as a mechanism of action of several therapeutic agents eg. Colloid Bismuth Subcitrate (Konturek et al, 1987) antacids and Ranitidine (Rachmilewitz et al, 1986).

Prostaglandins do not completely protect the gastric mucosa against necrotising agents. It appears that the superficial layers of the epithelium undergo extensive damage but that the deeper layers are protected

(Tarnawski et al, 1985). Within the deeper layers lies the cellular proliferative zone and since it is protected, regeneration occurs.

"Cytoprotection" has now been replaced by the more appropriate term "gastric mucosal protection". This involves many mechanisms:-

(a) Stimulation of mucus and bicarbonate secretion:

In man the pH of the gastric mucosal epithelium is maintained at around pH 7 despite an intra-luminal pH of 1 - 2 (Bahari et al, 1982). This involves the secretion of a glycoprotein-rich mucus gel which acts as an unstirred layer. It is negatively charged and retards H^+ and pepsin from diffusing back towards the cellular surface (Williams and Turnberg, 1980). Prostaglandins have been shown to stimulate mucus production (Johansson and Kollberg, 1979; Bolton et al, 1978).

Bicarbonate is secreted by epithelial cells and is trapped between the mucus layer and the cell surface. It neutralises acid which enters the mucus layer, converting it to CO_2 and water. Prostaglandins have been shown to increase bicarbonate secretion (Rees et al, 1984; Feldman, 1983; Isenberg et al, 1986). Non-steroidal anti-inflammatory drugs have the opposite effect. Mucus is important in protecting the gastric mucosa; Dekanski et al (1975) showed an inverse relationship between the amount of mucus and the extent of drug-induced erosions in rats. The mucolytic agent N-acetyl cysteine can

counteract the protective activity of mucus (Zlotoff et al, 1982). Acetazolamide (which inhibits gastric mucosal bicarbonate secretion) has a similar effect (Kollberg et al, 1981).

(b) Effect on adenyl cyclase:

Soll et al (1978) found that prostaglandin E stimulates the formation of cAMP in non-parietal cells. Many of these cells are located on the mucosal surface and release mucus into the lumen. This contrasts with the effect of PGE on the parietal cell where PGE reduces intra-cellular cAMP and blocks hydrogen ion secretion.

(c) Sodium pump:

Sodium is normally pumped from the luminal side to the serosal side of gastro-intestinal cells. This is enhanced by 16,16-dimethyl PGE₂ (Bowen et al, 1975), and reversed by indomethacin (Chaudhury and Jacobson, 1978). In injured cells this sodium flux is impaired and anions accumulate intracellularly, leading to retention of water, oedema and cell necrosis.

(d) Mucosal barrier:

Several prostaglandins have been shown to protect the mucosal barrier. This is another concept which is not fully understood but prostaglandins prevent alterations in pH across the cell wall caused by aspirin, indomethacin and ethanol (Cohen et al, 1975; Tepperman et

al, 1978). The mechanism of this protection has not been elucidated.

(e) Gastric mucosal circulation:

Doses of PGE_1 , PGE_2 , and PGI_2 which suppress gastric acid secretion also increase gastric mucosal blood flow whereas $\text{PGF}_{2\alpha}$ decreases gastric mucosal blood flow in animals (Broughton-Smith et al, 1978; Konturek et al, 1979).

(f) Repair mechanisms:

It has been observed that prostaglandins do not prevent injury to the superficial mucosa but protect the deeper layer where cell proliferation occurs (Tarnawski et al, 1985). This allows prompt re-epithelialisation of the denuded mucosa. PGE has been shown to enhance DNA, RNA, protein and collagen synthesis in cutaneous wounds (Lupulescu, 1975) but this has not been demonstrated in the gastric mucosa (Miller et al, 1982). Long term treatment with prostaglandins does lead to gastric mucosal hyperplasia in both animals (Reinhart et al, 1983) and man (Tytgat et al, 1982).

Although gastric mucosal protection involves several mechanisms which can be demonstrated in vitro, their significance in vivo is far from clear.

4. DUODENAL ULCER AND PROSTAGLANDINS

Peptic ulcers occur because of an imbalance between mucosal protective factors and injurious luminal factors. The mucosal protective factors include the mucus layer, mucosal bicarbonate secretion, blood flow and the regenerative capacity of the proliferative zone (Fig 1:8). Local prostaglandins influence these, although other factors are also involved. The main injurious factors are acid, pepsin, bile reflux, stasis, NSAID's and alcohol. Since prostaglandins influence these protective mechanisms, and exogenously administered prostaglandins analogues heal ulcers, it is possible that a defect in prostaglandin metabolism may be an important aetiological factor in peptic ulcer disease.

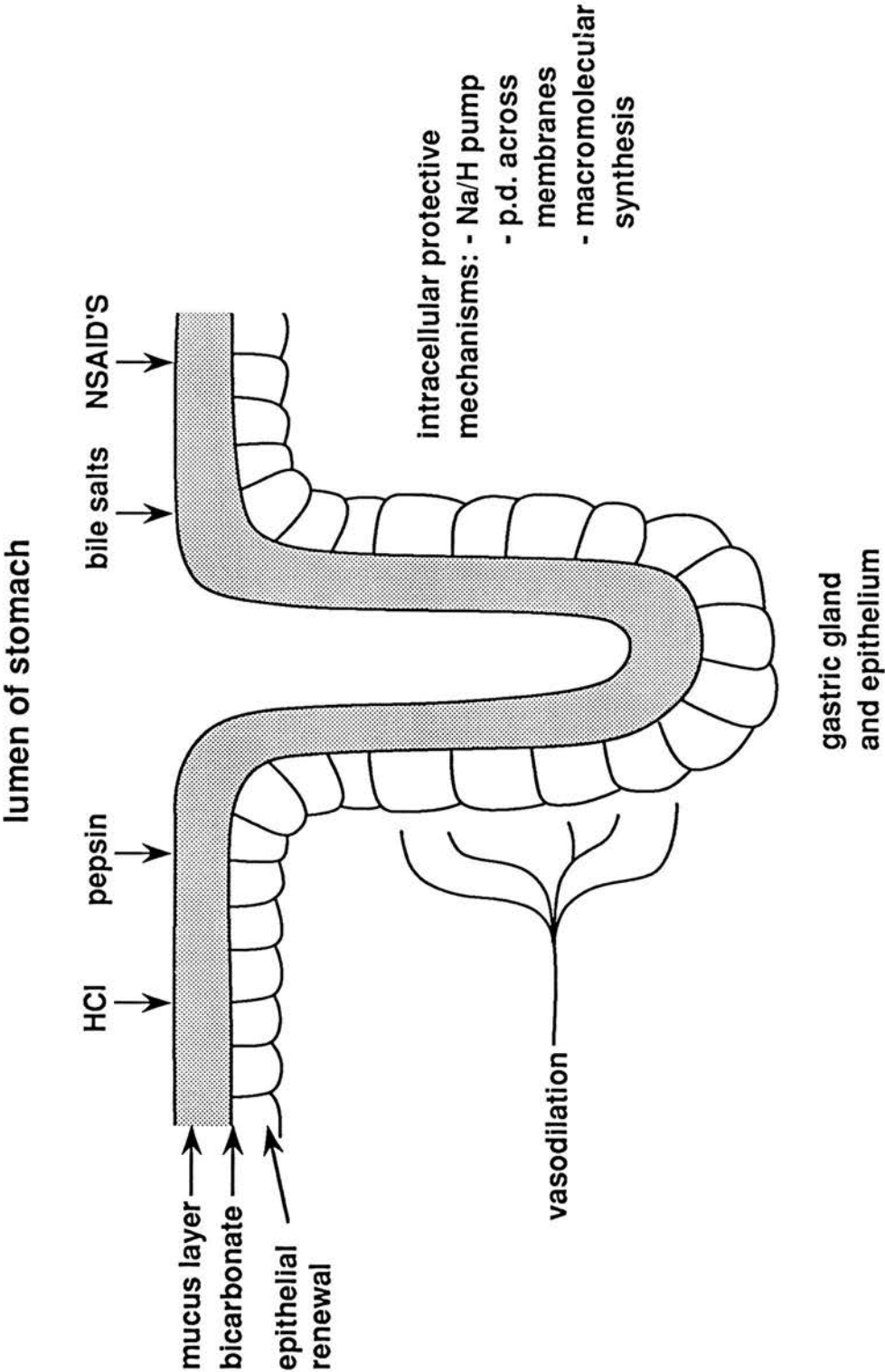
This hypothesis has been studied in three ways :-

1. Several groups have directly examined the synthesis of prostaglandins in mucosal biopsies from the antrum and duodenum. Others have looked at the output of prostaglandins in gastric juice. The findings are confusing and in some cases conflicting and are discussed fully in Chapter 2.

2. An alternative approach is to inhibit gastroduodenal prostaglandin metabolism. There is accumulating evidence that NSAIDS are associated with the development of peptic ulcer, particularly when complicated by perforation or bleeding (Domschke and Domschke, 1984; Gillies and

Fig 1:8

Pathophysiology of duodenal ulcer
- aggressive and defensive factors -



Skyring, 1969; Piper et al, 1981; Jick, 1981). Furthermore, corticosteroids, which are associated with the development of peptic ulcer, inhibit phospholipase A₂ release.

3. The final approach has been to examine the therapeutic effects of prostaglandin in experimental peptic ulcers. Pre-treatment with the prostaglandin analogues Misoprostol (Cohen et al, 1985) and Arbabrostil prevent aspirin induced gastric erosions. Several clinical trials have shown the efficacy of these drugs in peptic ulcer (Van Trappen et al, 1982; Bardhan et al, 1984), although they may act by suppressing acid rather than by augmenting gastric mucosal protection (Hawkey and Walt, 1986).

CHAPTER 2

THE EFFECT OF LINOLEIC ACID ON GASTRIC PHYSIOLOGY

1. INTRODUCTION AND AIMS

2. MATERIALS AND METHODS

Subjects

Design

Analysis of biological fluids

- a) Gastric acid output
- b) Prostaglandin output
 - i) materials
 - ii) handling of samples
 - iii) methyloximation of gastric juice
 - iv) radioimmunoassay procedure
 - v) performance of radioimmunoassay
- c) Mucus output
 - i) assay
- d) Serum Gastrin concentration

3. RESULTS

- a) Gastric acid output
- b) Prostaglandin output
- c) Mucus output
- d) Serum gastrin concentration

4. DISCUSSION

- a) Methodology
 - i) prostaglandin assay
 - ii) mucus assay

- b) Prostaglandin output
- c) Effect of linoleic acid on prostaglandins
- d) Gastric acid output
- e) Gastric mucus output
- f) Serum gastrin concentration

1. INTRODUCTION

Chapter I discussed the evidence that essential fatty acids are the biological precursors of prostaglandins and that prostaglandins have important effects upon gastroduodenal physiology. In the series of experiments described in this chapter, the effects of dietary linoleic acid supplementation on gastric acid secretion, output of PGE in the gastric juice, mucus output and fasting serum gastrin concentration were studied in healthy controls and in patients with duodenal ulcer disease. Hollander and Tarnawski had previously demonstrated in rats that dietary supplementation with arachidonic or linoleic acids induced an enormous increase in gastric prostaglandin concentration (Hollander et al 1982, 1985). PGE concentration increased from 880pg/ml to 4100ng/ml after supplementation - a 5000-fold increase. Schepp et al (1988) also showed that rats fed diets deficient in linoleic acid exhibited decreased gastric PGE secretion and this could be overcome by dietary linoleic acid supplementation.

No studies examining the relationship between dietary essential fatty acids ingestion and gastric PGE secretion had been performed in man. This is of interest because it relates to the hypothesis suggested by Hollander and Tarnawski (1986), that the decreasing virulence and incidence of peptic ulcer disease might be related to

increased essential fatty acid ingestion. If dietary linoleic acid favourably affects the gastroduodenal luminal milieu, it is possible that dietary manipulation might affect the natural history of duodenal ulcer disease.

In order to discriminate between the specific effects of linoleic acid (a poly-unsaturated fatty acid) and any non-specific effect of fatty acids, control experiments using the saturated fatty acid stearic acid were performed.

The aims of the experiments described in this chapter were :

1. To compare gastric prostaglandin, acid and mucus secretion and fasting serum gastrin concentration in duodenal ulcer patients with normal subjects.
2. To determine the effects of dietary linoleic acid upon gastric physiology - gastric prostaglandin output, gastric acid secretion, mucus output and fasting serum gastrin concentration.

2. MATERIALS AND METHODS

Subjects

14 healthy male volunteers aged 20 - 41 years (mean 31) were studied. All were non-smokers, none abused alcohol nor took any medications. None had dyspepsia and peptic ulcer was excluded by endoscopy. Nine were studied before and after a period of dietary supplementation with

linoleic acid. The remaining five subjects were studied before and after dietary stearic acid.

Seven patients aged 29 - 69 years (mean of 42 years) with endoscopically proven duodenal ulcer were studied. Their ulcers had healed following a course of H₂ receptor antagonists and all had completed treatment at least 3 weeks prior to the study. Endoscopy confirmed that the ulcers had healed. None abused alcohol, 3 smoked (10 - 15 cigarettes per day) but did not smoke during the study period. No drugs other than linoleic acid were taken during the study.

Design

Each subject was studied over a 14 day period (fig 2:1). On entry to the study a 10 ml venous blood sample was taken for measurement of fasting serum gastrin concentration. Endoscopy was performed using an Olympus GIF P10 forward viewing instrument. Intravenous sedation was not given although the pharynx was sprayed with Lignocaine spray. A size 10 FG nasogastric tube was then positioned in the stomach and after aspiration of resting juice, the gastric contents were continuously aspirated using a vacuum pump. At 15-minute intervals residual gastric juice was aspirated manually using a syringe. Three or four 15-minute basal samples were obtained, 0.6ug/kg of Pentagastrin (Peptavlon, ICI) was injected intramuscularly and a further four 15-minute aspirates were collected.

fig 2 :1

PROTOCOL OF STUDY

DAY 1

DAY 14

ENDOSCOPY
PENTAGASTRIN
BIOPSIES
FASTING GASTRIN
MUCUS
ROUTINE BIOCHEMISTRY

ENDOSCOPY
PENTAGASTRIN
BIOPSIES
FASTING GASTRIN
MUCUS
ROUTINE
BIOCHEMISTRY

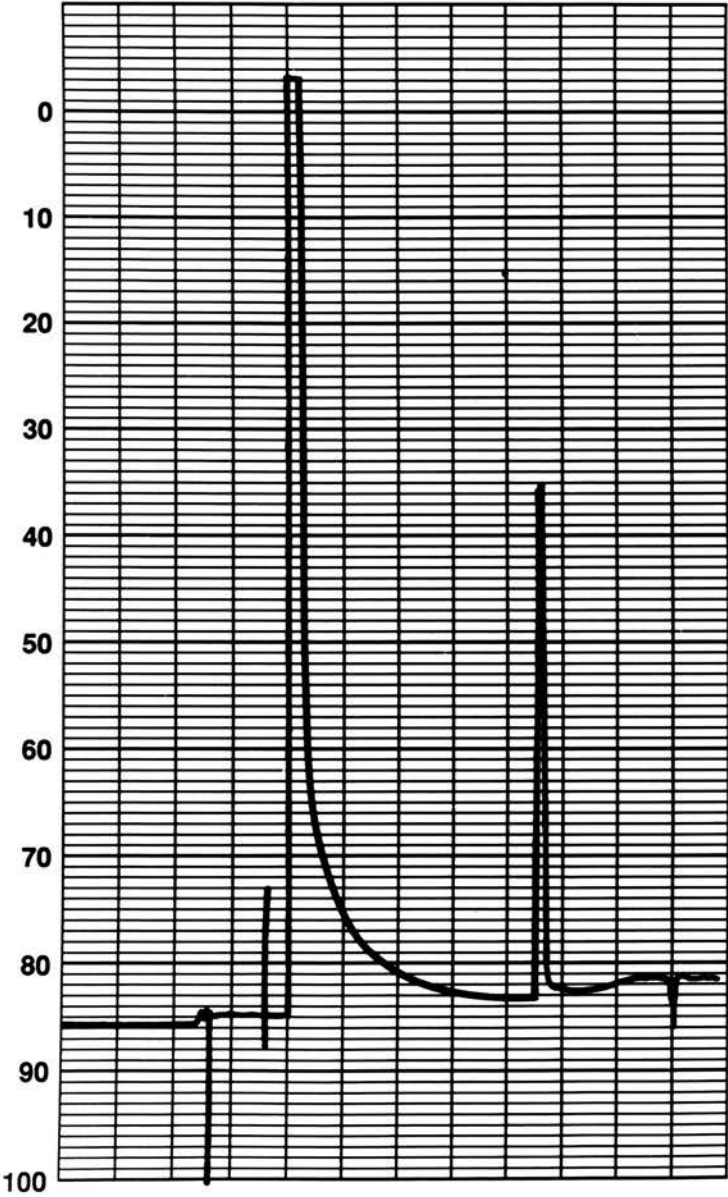
Each subject was studied before and after a 14-day period of dietary fatty acid supplementation to their normal diet. Five normal subjects took 1.5g of linoleic acid per day (in 3 divided doses), four normal subjects received 3g of linoleic acid per day (in 3 divided doses) and five control subjects took 3g of stearic acid per day. All the ulcer subjects received 3g of linoleic acid per day.

One hour after taking the last dose of fatty acid each subject was studied in exactly the same manner as on entry to the study; blood was taken for gastrin levels, endoscopy was performed to confirm normal appearances of stomach and duodenum, basal and pentagastrin stimulated gastric juice was collected.

Linoleic acid was prepared as a micellar suspension in the non-ionic detergent Pluronic F68. The product was administered in a gelatin capsule which dissolves in the acid medium of the stomach. Identity and purity were confirmed repeatedly over the period of the experiments by gas chromatography - mass spectrometry (GC-MS). Fig 2:2 depicts a representative trace. Samples of stearic acid were subjected to similar analysis. Experiments in animals had previously shown that Pluronic F68 has no effect upon gastric prostaglandin secretion (Tarnawski et al 1985).

Fig 2:2

VERIFICATION OF CONTENT OF LINOLEIC ACID CAPSULES
BY GAS CHROMATOGRAPHY- MASS SPECTROMETRY (GC- MS)



injection of
sample into
GC-MS

Linoleic acid
peak

Analysis of biological fluids

Gastric juice was analysed for acid, prostanoids and mucus. Serum samples were analysed for gastrin.

a) Gastric acid output

Two 5 ml aliquots of gastric juice were taken from each 15-minute period of gastric aspiration for measurement of hydrogen ion concentration. This was measured in duplicate and mean values calculated.

Acid concentration was determined by back titration to pH7 with 0.1N NaOH using an automated analyser (PH M82 pH meter, Radiometer, Copenhagen). Hydrogen ion output was calculated for each 15-minute period from the product of concentration and volume. The values determined before pentagastrin will be referred to as "basal" acid output, those following pentagastrin as "maximal" acid output.

b) Prostaglandin estimation

PGE and PGEM concentrations were measured using radioimmunoassay.

(i) Materials Tritiated prostaglandins were obtained from Amersham International (Bucks) and New England Nuclear (Stevenage). Pure standard prostaglandins were purchased from Sigma (Poole, Dorset). Methyloximating

reagent was made from methoxyamine hydrochloride and anhydrous sodium acetate (obtained from Eastman Kodak).

Antibodies to PGE and PGEM were kindly donated by Dr R Kelly, MRC Reproductive Biology Unit, Edinburgh. They were obtained using rabbits as described by Kelly et al (1986).

(ii) Handling of gastric juice. Immediately after being collected the volume of gastric juice was measured. 10 mls was removed for measurement of acid concentration as previously described, and the pH of the remainder was adjusted to 7 to minimise conversion of PGE to PGA. Samples were then frozen and stored at -20°C.

(iii) Methyloximation Methyloximation of prostanoids increases their stability. A methyloximating solution was prepared by mixing 5g of methoxyamine hydrochloride, 41g of anhydrous sodium acetate, 50 mls of ethanol and 500 mls distilled water, the pH was then adjusted to 5.6.

2 mls of neutralised gastric juice were mixed with 2 mls of methyloximating solution and left to stand overnight. Methyloximating efficiency had previously been assessed by using tritium labelled 6-keto prostaglandin $F_{1\alpha}$ and carbon-14 labelled PGE_2 . HPLC separation before and after methyloximation showed 97% conversion for 6-keto $PGF_{1\alpha}$ and 96% for PGE_2 .

(iv) Radioimmunoassay procedure. Radioimmunoassay was performed in triplicate using a standardised technique. The protocol is illustrated in Tab 2:1 . A standard curve using commercially obtained PGE and PGEM was constructed. Concentrations ranged from 0.02-10 ng/tube.

"Non-specific binding" - each tube contained 100ul of tritiated label and 200ul of tris-EDTA buffer. As there was no antibody nor unlabelled antigen, any binding of the tritiated label was due to non-specific effects.

"Reference standard" or "maximum binding" - Each tube contained 100ul of tritiated label, 100ul of tris-EDTA buffer and 100ul of antiserum. There was tritiated antigen and antiserum but no unlabelled antigen so that binding of the labelled antigen was maximal.

"Standard curve" - For each assay a standard curve was run, and differing quantities of unlabelled prostaglandin ranging from 0.02ng to 10ng (in 100 ul) were added to 100ul of tritiated prostaglandin and 100ul of antiserum. When larger quantities of unlabelled prostaglandin were added relatively less of the tritiated prostaglandin bound to the antibody and the counts were lower, when smaller amounts of unlabelled prostaglandin were added more of the unlabelled prostaglandin bound to the antibody and the

table 2:1

PROTOCOL FOR PERFORMING RADIOIMMUNOASSAY

	³ H label antigen	tris EDTA buffer	anti- serum	standard curve	unknown antigen sample
TOTAL COUNTS	100	---	---	---	---
NON-SPECIFIC BINDING (NSB)	100	200	---	---	---
REFERENCE STANDARD	100	100	100	---	---
STANDARD CURVE	100	---	100	100	---
TEST SAMPLE	100	---	100	---	100
QUALITY CONTROL (QC)	100	50	100	50	---

ALL FIGURES ARE IN MICROLITRES

counts were high. The standard curve has a reverse sigmoid form; a typical example is shown in fig 2:3.

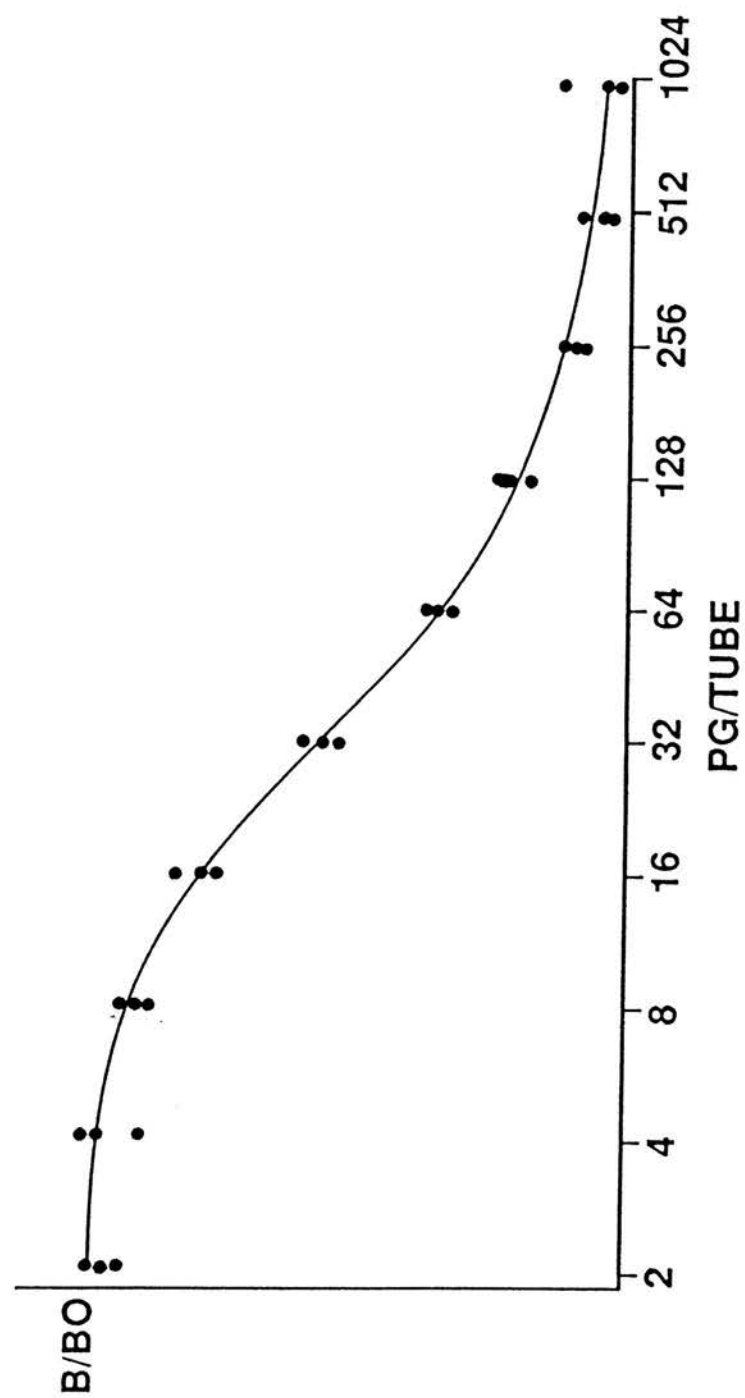
"Test samples" - 100ul of tritiated label, 100ul of antiserum and 100ul of the sample to be tested were added together. The amount of tritiated antigen bound to the antiserum can be counted and is inversely proportional to the amount of unlabelled antigen (sample) present. The quantity of antigen present in the sample is calculated with reference to the standard curve.

"Quality control" - 100ul of tritiated prostaglandin are added to 100ul of antiserum, 50ul of tris-EDTA buffer and 50ul of a known amount of unlabelled prostaglandin. The result gives an estimate of intra-assay precision.

Samples of methyloximated gastric juice were assayed in identical manner; 100 ul of tritiated label, 100 ul of antiserum and 100 ul of the sample being tested in triplicate. The Samples were vortex mixed and incubated for twelve hours at 4°C, covered by aluminium foil (to prevent evaporation). Once equilibrium had been attained 0.8 ml of polyethylene glycol (PEG) was added (forming a plug which remained in the tube). Tubes were centrifuged at 3000 rpm (1684 x G) at 4°C for fifteen minutes then inverted and allowed to drain for fifteen minutes to enable all the unbound prostaglandins to be separated. One ml of scintillant (RIA LUMA) was added to

Fig 2:3

Typical standard curves of PGE



each tube, the tubes were shaken to dissolve the pellet and then placed in a self loading β -counter for one minute (1216 Rackbeta liquid scintillation counter, LKB Wallac, Milton Keynes, England).

Attention was paid to detail, contact with other workers using prostaglandins was avoided and the risk of contamination of utensils was avoided by using disposable equipment, automatic dispensers and pipettes.

(v) Performance of the radioimmunoassay

Specificity: the specificity of the assay had previously been determined by Kelly et al. There was almost no cross-reactivity between the PGE radioimmunoassay and PGA, PGEM, 6-keto PGF_{1 α} and linoleic acid. There was 53% cross reactivity between PGE₁ and PGE₂ and 31% between PGE₃ and PGE₂. There was no cross-reactivity between the PGEM assay and PGA, PGE, 6-keto PGF_{1 α} and linoleic acid.

Sensitivity: the sensitivity of both the PGE and PGEM assay was 20 pg/100 μ l in gastric juice.

Precision: the intra-assay precision of PGE ranged from 13.5 - 26.4%. That of PGEM was 12.1 - 22.3% (relative SD). Inter-assay precision of PGE was 14.8%; that of PGEM was 9.4% (relative SD).

Reproducibility: reproducibility was assessed by

repeated assays on the same specimens. In representative samples the correlation coefficient was 0.98. A similar value (0.78) was obtained by an independent worker using the sample (Figs 2:4 and 2:5).

c) Mucus output

Quantifying mucus gel secretion poses several methodological problems as it is necessary to distinguish between adherent mucus and soluble mucus. Initially an attempt was made to measure the adherent mucus layer using a pachometer modified from a method described by Douthwaite and Spence (1986). The method was found to be unsuitable in this study because only small biopsies were obtainable from the gastric mucosa resulting in crushing, crumpling and distorting of the mucus layer. The technique was therefore abandoned.

i) Assay

Soluble mucus output was measured using the method described by Baron et al (1986). This assumes that mucus comprises the vast majority of the solid component of the gastric juice. The aqueous components are removed by dialysis and lyophilisation, then homogenised to disperse any insoluble mucus, and finally centrifuged at 2000G for ten minutes and the volume measured. The gastric juice was concentrated by rotavapour (when

Fig 2:4

CORRELATION BETWEEN 2 ASSAYS FOR PG E

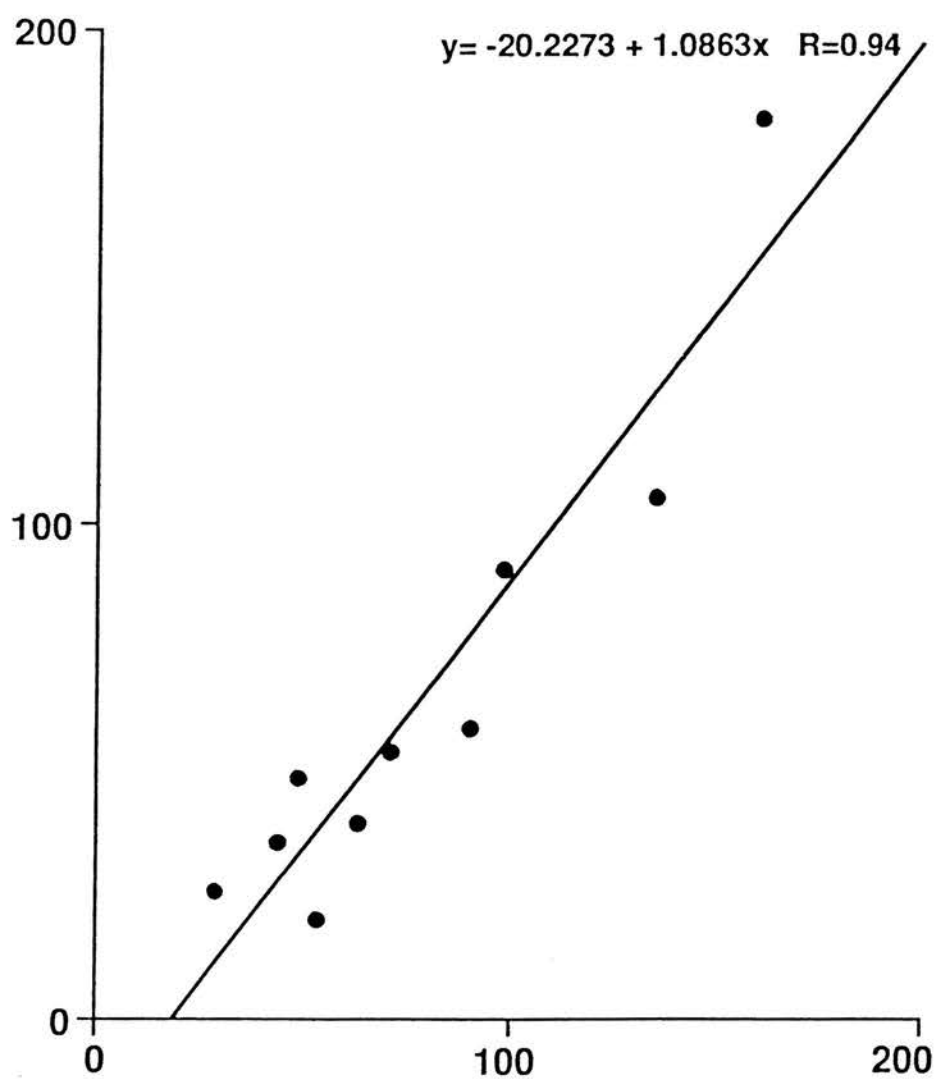
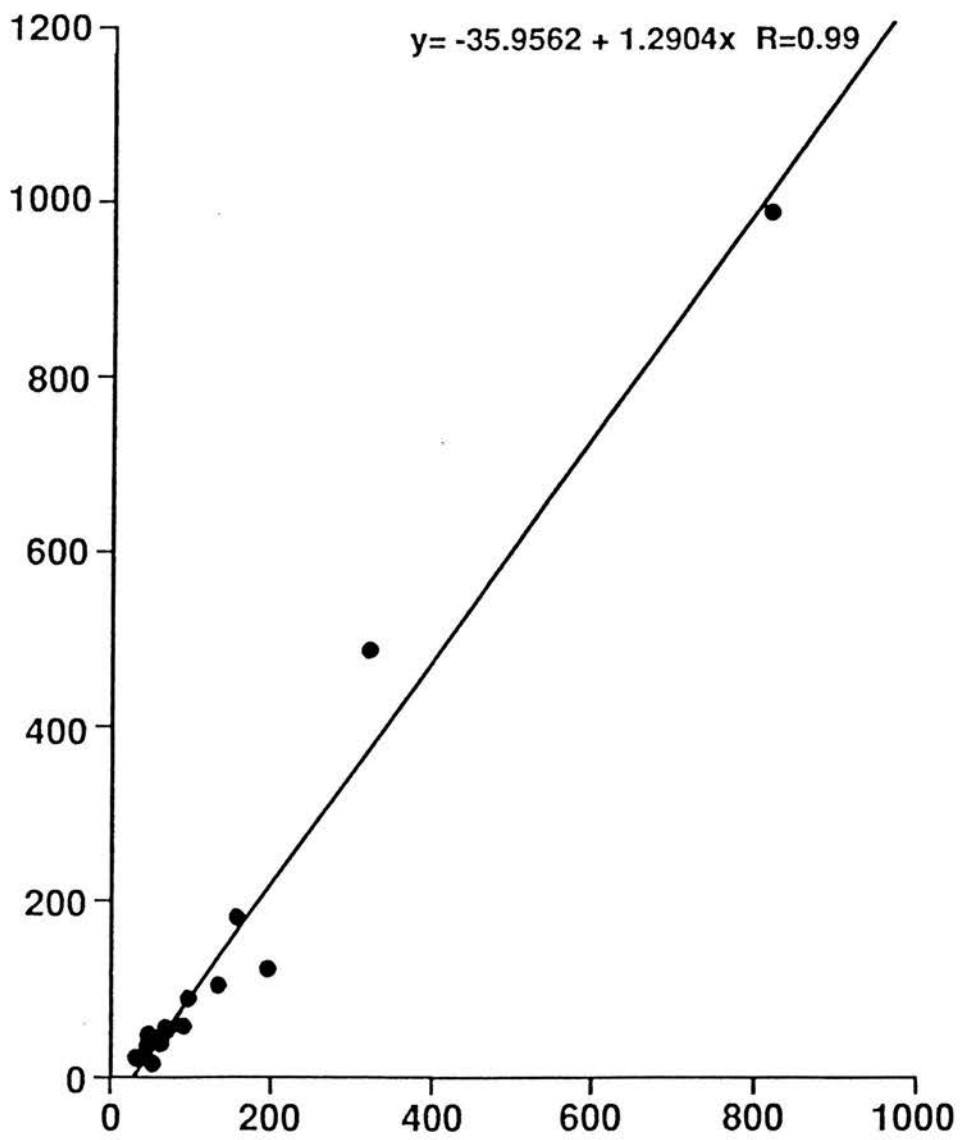


Fig 2:5

CORRELATION BETWEEN 2 ASSAYS FOR PG EM



necessary) and dialysed with distilled water for seventy two hours (five changes of the dialysis fluid). The mucus output was calculated by multiplying the concentration of non-dialysable material in the sample of gastric juice by the corrected volume of the juice produced in the basal and stimulated hours.

d) Serum gastrin concentration

After an overnight fast 10 ml of venous blood was removed from the subject, the sample was spun down immediately, the serum removed and stored at -20° . The serum gastrin concentration was measured using radioimmunoassay by the Department of Biochemistry, Glasgow Royal Infirmary. Detection limit of the assay was 15 ng/l, the within batch co-efficient of variation was 7% and the between batch variation was 12%. The anti-serum reacts with gastrins containing 17 and 34 amino acids and to a lesser extent with big, big gastrin.

3. RESULTS

Endoscopy, collection of gastric juice and ingestion of the fatty acids were well tolerated. Two control subjects receiving linoleic acid developed mild diarrhoea (loosening of stool without increased frequency) but

there were no other side effects associated with either linoleic or stearic acid ingestion. Compliance was determined by interview, counting of capsules and by the observation of partially broken down capsules with blue discolouration at endoscopy. Endoscopic appearances were all normal or showed ulcer scars in the ulcer group.

a) Gastric acid output

The results of gastric acid secretion are presented in tab 2:2 and Fig 2:6. In the 9 healthy controls receiving linoleic acid mean basal acid output was 6.2 mmol/hr (± 1.5) (SEM) before linoleic acid and 5.3 mmol/hr (± 1.2) after linoleic acid ($p > 0.05$). Mean maximal acid output fell from 35.1 mmol/hr (± 3.5) prior to linoleic acid to 30.0 mmol/hr (± 2.7) after linoleic acid ($p < 0.05$).

In the 7 patients with duodenal ulcer mean basal acid output was 3.0 mmol/hr (± 0.8) before linoleic acid and 1.8 mmol/hr (± 0.6) after linoleic acid ($p > 0.05$). Mean maximal acid output was 29.2 mmol/hr (± 2.3) before linoleic acid and 28.5 mmol/hr (± 2.7) after linoleic acid ($p > 0.05$).

In the 5 healthy controls taking stearic acid mean basal acid output was 3.8 mmol/hr (± 1.7) and 1.8 mmol/hr (± 1.1) after stearic acid. Mean maximal acid output was 25.0 mmol/hr (± 2.3) before stearic acid and 23.7 mmol/hr (± 1.4) after stearic acid ($p > 0.05$). There was no significant change in acid output in this control

Tab 2:2

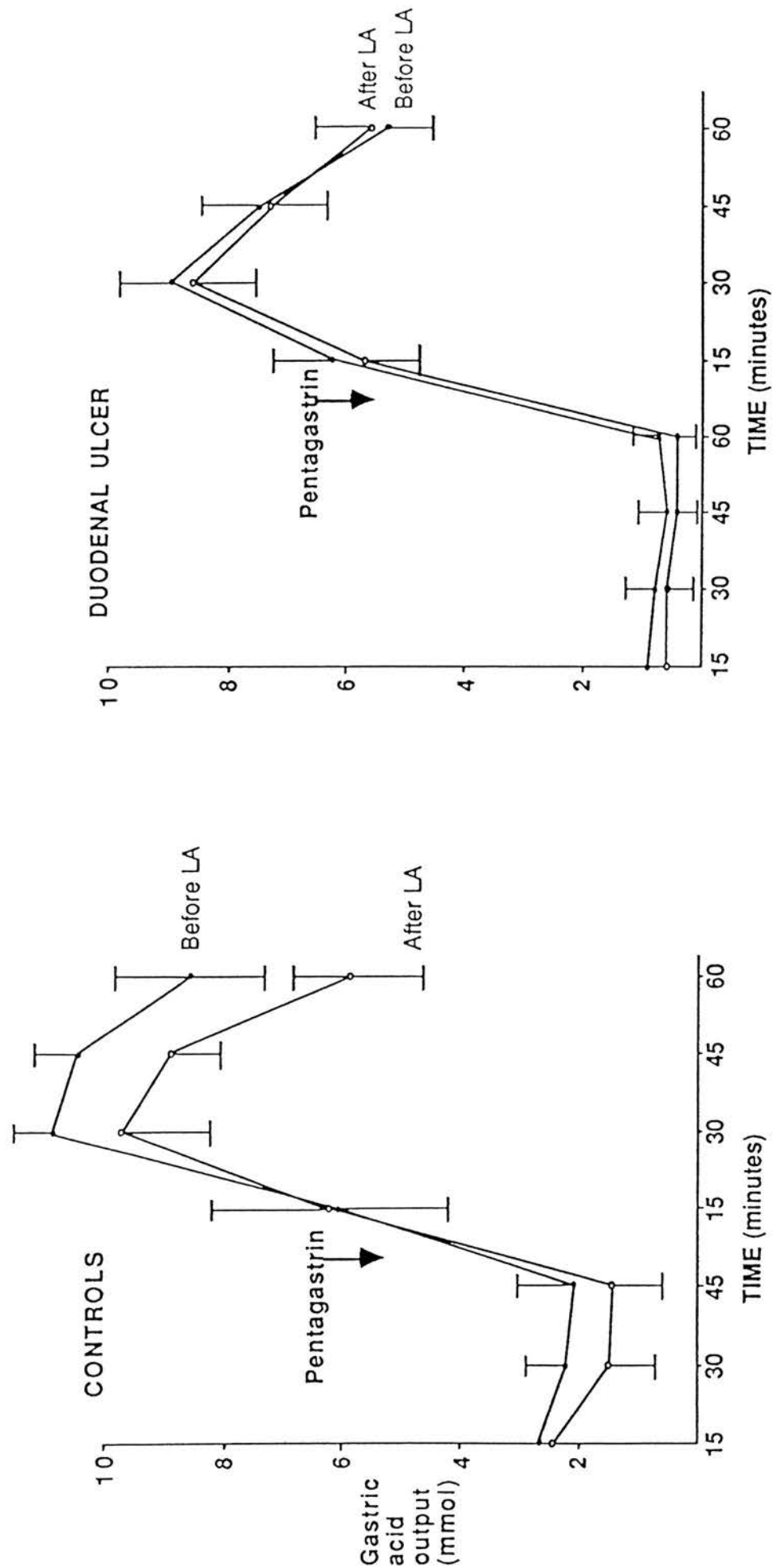
Mean (\pm SEM) gastric acid output in control and duodenal ulcer subjects before and after dietary supplementation with linoleic or stearic acid

	Basal acid output mmol/hr		Maximal acid output mmol/hr	
	Before	After	Before	After
controls taking linoleic acid n=9	6.2 (1.5)	5.3 (1.2)	35.1 (3.5)	30.0 (2.7) *
DU patients taking linoleic acid n=7	3.0 (0.8)	1.8 (0.6)	29.2 (2.3)	28.5 (2.7)
controls taking stearic acid n=5	3.8 (1.7)	1.8 (1.1)	25.0 (2.3)	23.7 (1.4)

* = differs from pre-linoleic acid value ($p < 0.05$)

Fig 2:6

MEAN GASTRIC ACID OUTPUT (\pm SEM) BEFORE AND AFTER LINOLEIC ACID



group indicating that the observed significant increase after linoleic acid is not a non-specific effect of fatty acids.

b) Prostaglandin output

The results of gastric PGE and PGEM output are presented in Tab 2:3 and in figs 2:7 and 2:8. Mean immunoreactive PGE output was significantly lower in ulcer subjects than controls ($p < 0.01$). In the controls, but not ulcer subjects, PGE output increased significantly following dietary linoleic acid. Mean PGEM was significantly higher in the ulcer patients than in the control group prior to linoleic acid (fig 2:9). The ratio of PGE:PGEM was reversed in control and ulcer subjects (PGE:PGEM ratio of approximately 2.5:1 in normal subjects and 1:6 in the ulcer group). Following linoleic acid PGEM output increased in the normal subjects and the magnitude of this increase was greater than that of PGE. The PGE:PGEM ratio approached unity after linoleic acid. In ulcer subjects there was no increase in mean PGEM output and the ratio of PGE : PGEM was unchanged following linoleic acid .

Stearic acid did not affect PGE or PGEM output in the normal subjects. The absolute levels of PGE and PGEM were lower than in the control group taking linoleic acid because there was a delay before they could be assayed.

Tab 2:3

Mean (\pm SEM) PGE and PGEM output in control and duodenal ulcer patients before and after dietary supplementation with linoleic acid or stearic acid

	PGE output ng / hour		PGEM output ng / hour	
	Before	After	Before	After
controls taking linoleic acid n = 9	498 (110)	1230 (465) *	192 (19)	1335(708) *
DU patients taking linoleic acid n = 7	138 (41) **	96 (21) **	840 (439) **	600 (173) **

* = differs from value before linoleic acid ($p < 0.01$)

** = differs from corresponding values in controls ($p < 0.01$)

Fig 2:7 MEAN GASTRIC PGE (\pm SEM) OUTPUT BEFORE AND AFTER LINOLEIC ACID

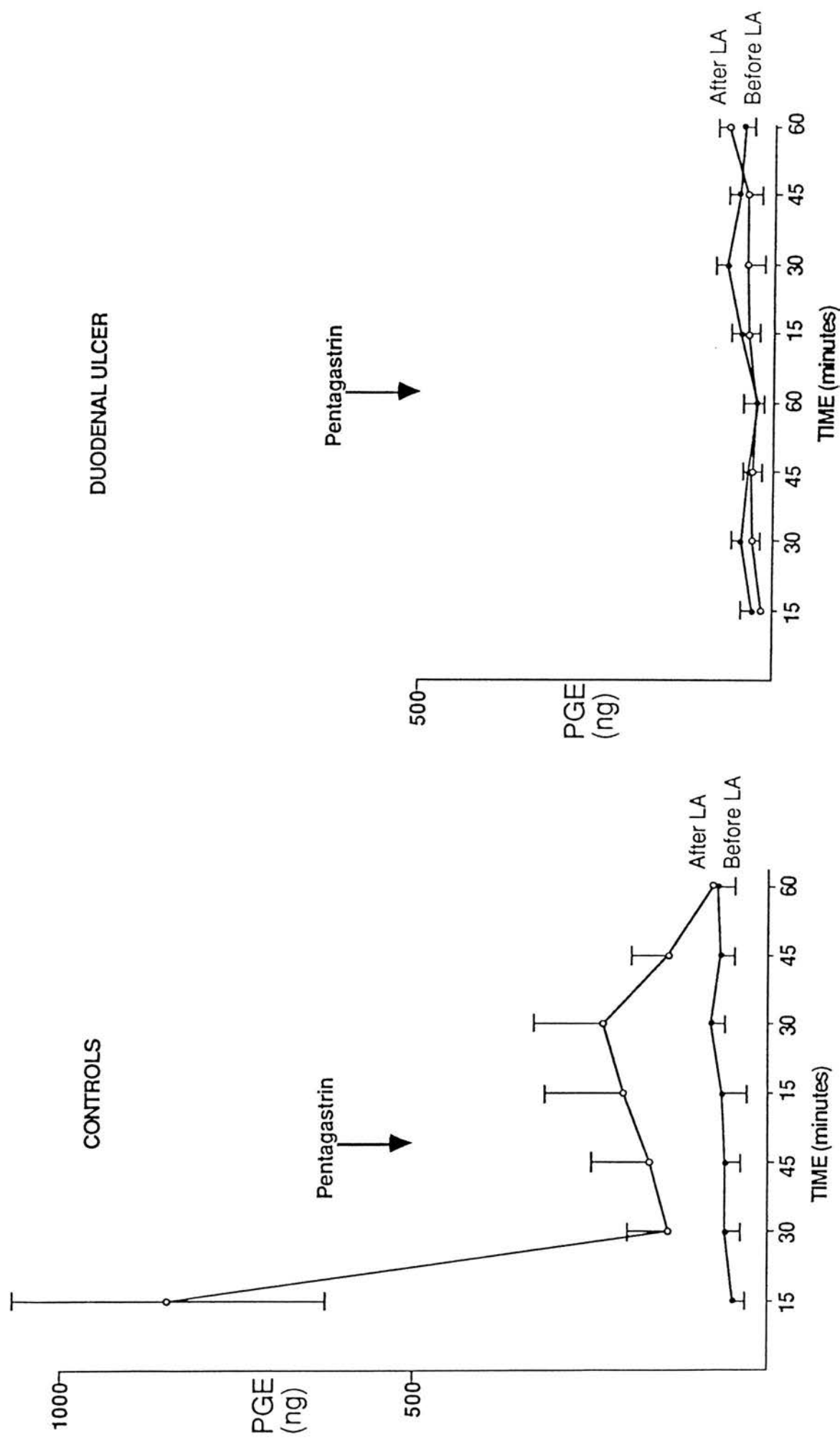


Fig 2:8

MEAN GASTRIC PGEM (\pm SEM) OUTPUT BEFORE AND AFTER LINOLEIC ACID

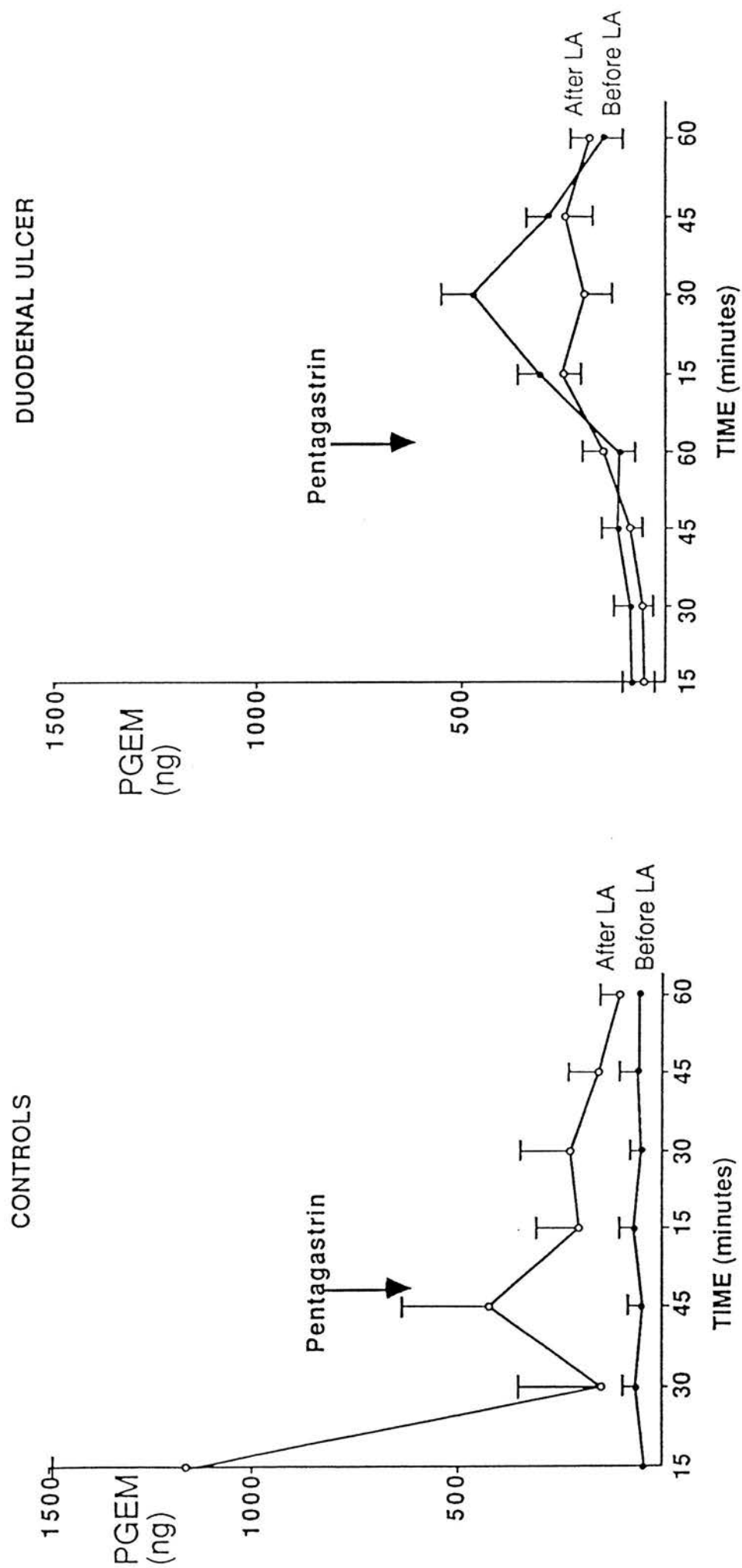
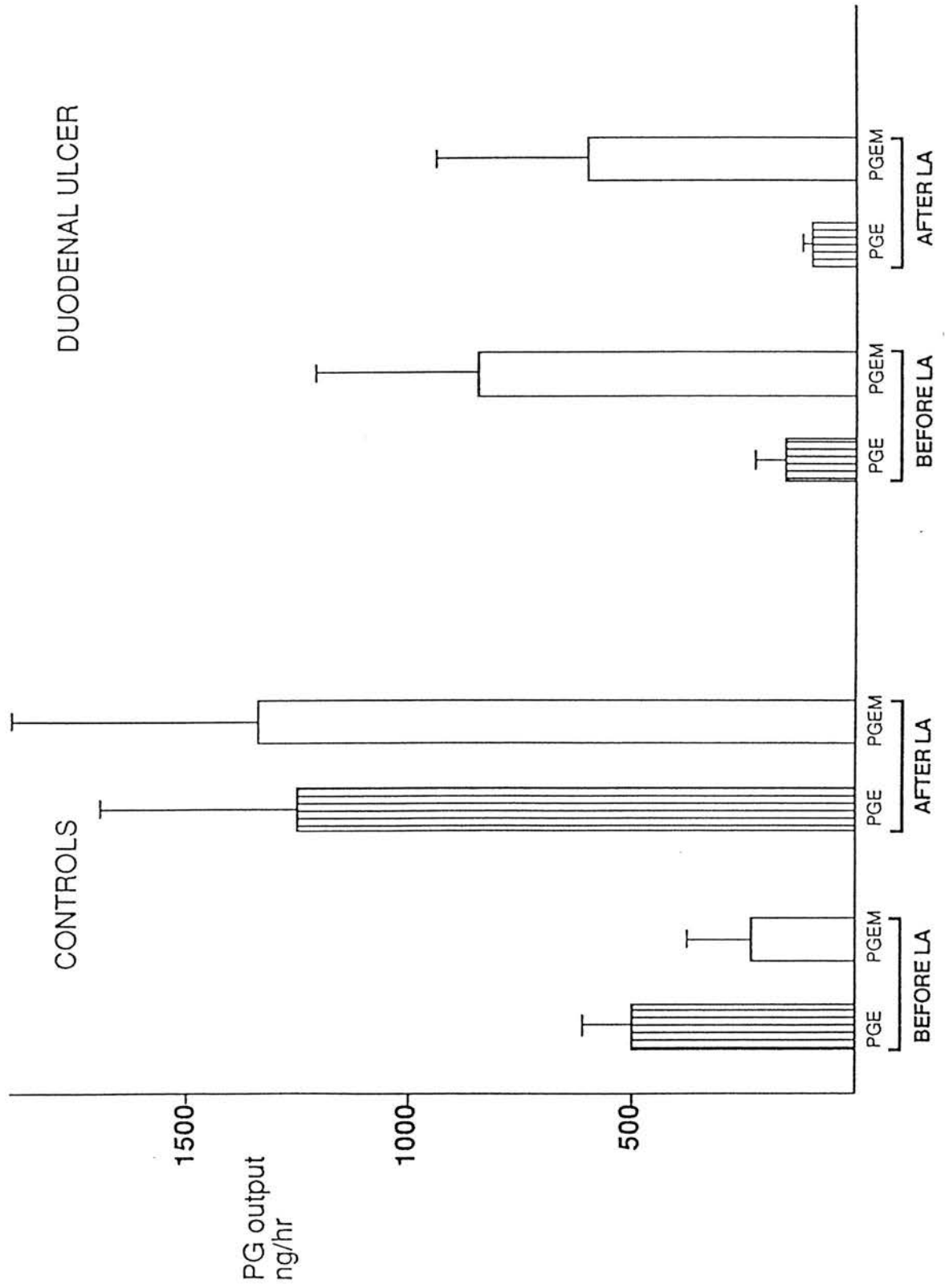


Fig 2:9 MEAN PGE AND PGEM OUTPUT (ng/hr) IN CONTROL AND DU PATIENTS



c) Mucus output

The results of mucus output are shown in Table 2:4. In the controls there was a significant increase in the output of mucus from 432 mg/hour (± 47) before linoleic acid to 593 mg/hour (± 83) after linoleic acid. In contrast, there was no significant difference in mucus output in the duodenal ulcer patients: it was 367 mg/hour (± 80) before linoleic acid and 388 mg/hour (± 95) after linoleic acid ($p < 0.05$).

d) Serum Gastrin Concentration

In control subjects mean fasting serum concentrations increased from 19.2 ng/l (± 3.3) (SEM) to 30.9 ng/l (± 4.1) following dietary linoleic acid ($p < 0.01$). This is shown in fig 2:10 where it can be seen that in all but one subject the mean serum gastrin concentration increased.

In ulcer patients the mean serum gastrin concentration did not significantly change following linoleic acid; 33.4 ng/l (± 4.5) before linoleic acid falling to 26.0 ng/l (± 3.5). This is shown in fig 2:10.

In control subjects receiving stearic acid there was also no significant difference in gastrin concentration before or after fatty acid supplementation; it was 25 ng/l (± 4.0) before stearic acid and 18 ng/l (± 3.8) after.

Tab 2:4

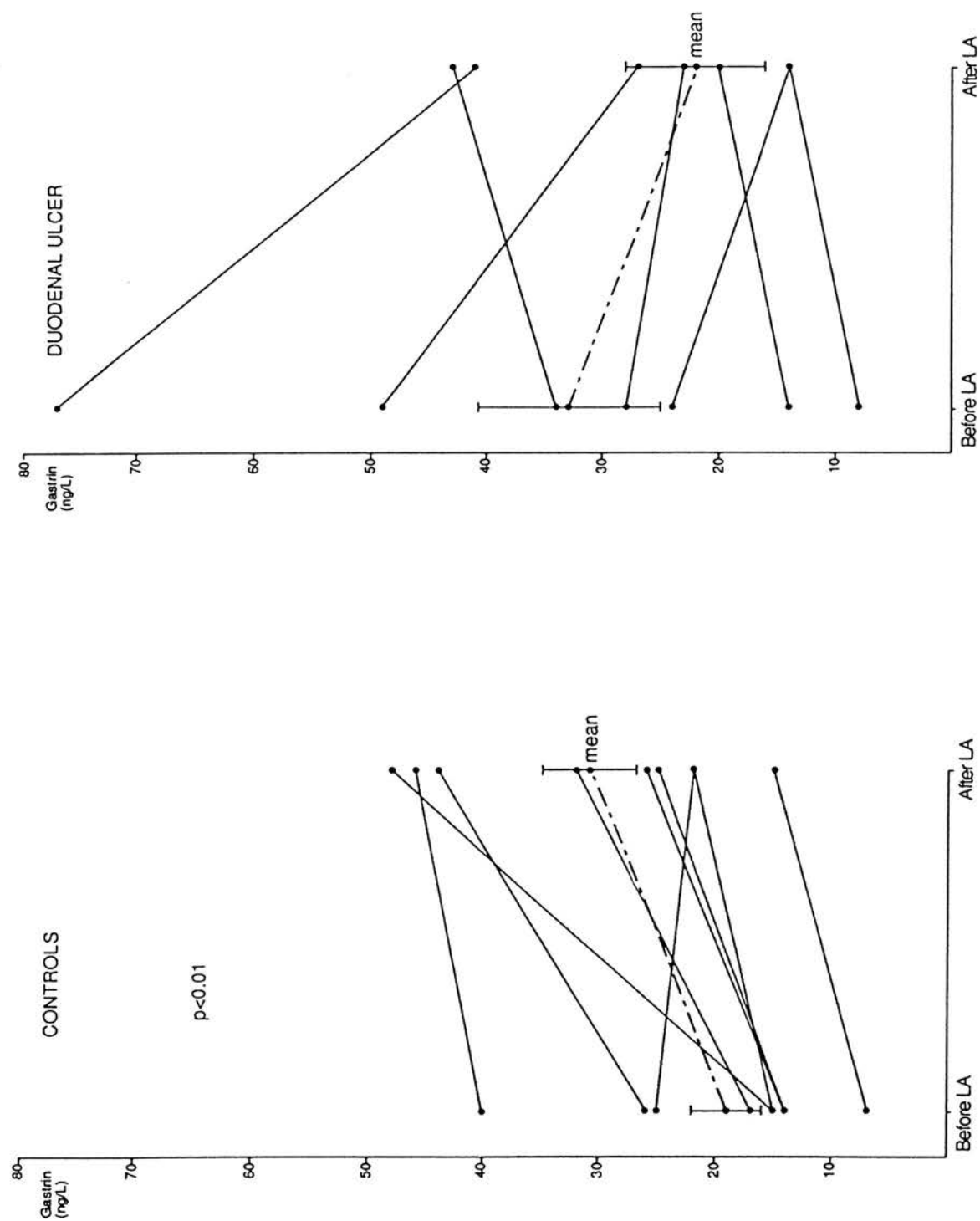
Mean (\pm SEM) mucus output in control and duodenal ulcer subjects before and after dietary supplementation with linoleic acid

	Mucus output (mg/ hr)	
	Before LA	After LA
Controls n = 9	432 (47)	593 [*] (83)
Duodenal ulcer n = 7	367 (80)	388 (95)

* = differ from value before linoleic acid (p < 0.05)

results not available for stearic acid

Fig 2:10 FASTING SERUM GASTRIN CONCENTRATION BEFORE AND AFTER LINOLEIC ACID



4. DISCUSSION

a) Methodology

(i) Prostaglandin assay

Prostaglandins are notoriously unstable; prostacyclin has a half-life of thirty seconds before being converted to 6-keto $F_{1\alpha}$, PGE is rapidly converted to PGA in an acidic environment, and PGA is rapidly converted to PGC in alkaline media. In order to minimise these problems samples of gastric juice were neutralised as soon as possible after being aspirated and samples were derivatised to their methyloxime. Methyloximation is common in gas chromatography-mass spectrometry (Kelly R et al, 1983). The technique has also been described for radioimmunoassay (Kelly and Abel, 1986) but is not in widespread practice. The process is simple, the reaction occurs at room temperature, and specific and sensitive antisera can be raised against the prostaglandins as their methyloximes.

Some workers extract prostaglandins from gastric juice prior to radioimmunoassay. The main reasons in favour of extraction are: to concentrate the prostaglandin, to dissociate the prostaglandin from any protein present, to remove any interfering factors, and to remove any other interfering prostaglandins if the antiserum is not particularly specific.

In this study it was decided not to extract

prostaglandins for several reasons: the level of prostaglandins in gastric juice is high and can be measured directly, it was possible to increase the sensitivity of the assay using a labelled ligand of higher specific activity , and omitting extraction avoids the problem of estimating recovery. This can be a problem as recovery can be low, variations occur, and non-specific interfering factors can be introduced. In a series of preliminary experiments (tab 2:5) it was shown that for the concentrations found in gastric juice, this step was unnecessary. Using the Vac-Elut extraction system results for PGE output were almost identical to the unextracted values.

Assaying the main metabolite of PGE - 13,14 dihydro 15-keto PGE₂ gives an indirect marker of any PGE which may have been missed by the assay. PGA was not assayed in this study because the only antiserum available had unacceptably high cross-reactivity with other prostaglandins.

(ii) Mucus assay

It is difficult to measure adherent mucus and only the soluble mucus fraction was measured. The method used is relatively crude, assuming that all the non-dialysable component of the gastric juice is mucus. In fact this appears reasonable since the only other large molecular

Table 2:5

Results after Prostaglandin extraction

trace result	% of total	fraction
6712	100	prior to extraction
5934	88	full supernatant
41	0.7	straight through column
38	0.6	water wash
68	1	hexane wash
6625	99	ethyl acetate

Tracer was added to gastric juice and spun down. The supernatant was then added to the "Vac - elut" column. Most of the prostaglandin adheres to the column, and the small amount that goes through is counted. Ethyl acetate is poured into the column: this removes the prostaglandins which have adhered to the column, and the effluent is counted. This value represents the quantity of extracted prostaglandin. The results demonstrate that extraction of the samples is not necessary.

components are digestive enzymes, present in relatively low concentrations.

Mucus swallowed from the upper respiratory tract may have produced some artifactual increase in mucus output since the pharynx was not aspirated during the experiments. Since the amount of swallowed mucus is probably similar for the same individual studied on two occasions this is unlikely to have significantly affected the conclusions of the experiments.

The only solid material which may have influenced apparent mucus secretion was the gelatine capsule of the fatty acid capsule but this was either removed prior to dialysis or had passed through the pylorus before the juice was collected.

b) Prostaglandin output

This study has shown striking differences in gastric prostaglandin metabolism between normal subjects and patients with duodenal ulcer. Mean gastric PGE output was markedly lower in the duodenal ulcer patients; the finding of increased PGEM output strongly suggests that this was due to increased catabolism of PGE, rather than to decreased PGE formation. In normal subjects there was a marked rise in gastric output of PGE following linoleic acid, but this did not occur in ulcer patients.

The present observations contradict those of Cheung

et al (1976), who reported increased prostaglandin output in gastric juice from a small number of patients who had active duodenal ulcers. The presence of active ulceration may have resulted in non-specific rises of prostaglandin output masking an inherent abnormality, because tissue damage and inflammation stimulate prostaglandin metabolism and lead to increased PGE output. By studying patients whose ulcers were in remission, abnormalities caused by the non-specific effects of tissue damage were avoided.

Several groups have directly examined gastro-duodenal prostaglandin metabolism in health and peptic ulcer disease.

Sharon et al (1983) studied cultured duodenal and gastric mucosa in healthy and ulcer patients. Accumulation of PGE₂, 6-keto PGF_{1α} and TxB₂ from cultured duodenal mucosa in patients with active duodenal ulcers and in healthy controls was similar. However, accumulation of the same prostaglandins in cultured gastric mucosa was significantly less from patients with active duodenal ulcers than from healthy controls. The authors suggested that abnormal prostaglandin metabolism may be important in the pathogenesis of ulcer disease and suggest a possible role for exogenous prostaglandins.

Konturek et al (1981) found slightly reduced PGE₂-like activity from biopsies taken from the fundus and antrum of patients with acute duodenal ulcers compared to

healthy controls but this did not reach significance.

Crampton et al (1987) found no difference in PGE synthesis or degradation rates between duodenal ulcer and normal subjects. They did however find that gastritis was associated with increased prostaglandin E formation and that gastric ulcer patients had a reduced synthesis and degradation of PGE₂.

Ahlquist et al (1983) found no difference in the synthesis of PGE from the duodenal mucosa between duodenal ulcer patients and controls. However, after a meal normal subjects had a rise in the synthetic activity of prostanoids whereas in duodenal ulcer patients there was a fall in synthetic activity.

Pugh et al (1989) found that the synthesis of PGE₂ was reduced in patients with active and inactive duodenal ulcer disease. This decrease occurred both at the ulcer site and from morphologically normal duodenal and antral mucosa.

These studies all involved incubating mucosal biopsies and assessing their capacity to synthesise prostaglandins. This is open to artifactual disturbances since the trauma of biopsy, homogenisation and incubation non-specifically stimulates prostaglandin formation. In an attempt to overcome this, several groups have looked at the concentration of prostaglandins in gastric juice. Conflicting results have also been obtained using this

approach.

Hinsdale et al (1974) found that PGE levels in the gastric juice of patients with inactive duodenal ulcer were higher than in normal subjects under basal conditions. This difference disappeared after stimulation.

The confusion resulting from these studies may be due to the fact that insufficient attention was paid to the instability of prostaglandins, the non-specific prostaglandin stimulating effect of an active ulcer diathesis and failure to differentiate between changes in prostaglandin production and catabolism.

In this study the absolute levels of PGE in healthy controls were significantly higher than in the ulcer group ($P < 0.01$) (498ng/hr compared to 138ng/hr). In the healthy control group far less metabolite (192ng/hr) was found compared with PGE (498ng/hr), (fig 2:9). In contrast, in the ulcer group there was far more PGEM (840ng/hr) than PGE (138ng/hr). In the controls the ratio of PGE:PGEM was 2.5:1 whereas in the ulcer group it was 1:6. In this study PGE output was significantly lower in duodenal ulcer patients compared with controls whereas PGEM was significantly higher. When taken together the combined outputs of PGE and PGEM are similar in the 2 groups (690ng/hr in healthy controls, 978ng/hr in ulcer patients).

At first glance the lower PGE output ulcer patients seems at variance with other published series (Konturek et al, 1981; Ahlquist et al, 1983). However when PGE and PGEM are combined, output is similar in the 2 groups, which is in broad agreement with Sharon et al (1983) and Crampton et al (1987). The important difference demonstrated in this study is that in duodenal ulcer patients far more of PGE is found as its metabolite. This would suggest that in duodenal ulcer there is not an increase in the production of PGE as suggested by Hinsdale et al (1974) and Cheung et al (1976) but rather an increase in metabolism. The reason for this is not clear, although it could be due to an increase in activity of the enzyme endoperoxide isomerase. It is unlikely to be due to acid degradation of PGE to PGA because the acid outputs were similar in the two groups.

c) Effect of Linoleic acid on Prostaglandin output

Previous work in rodents by Tarnawski et al (1983, 1985) showed that intra-gastric linoleic acid and arachidonic acid resulted in enormous increases in PGE output (5,000 fold). Schepp et al (1988) reported similar findings. Some of this huge increase may have been due to cross-reaction of their PGE antibody with linoleic acid. This was not the case in this study where cross-reactivity was less than 0.001%. Furthermore, in the ulcer group no increase was found in PGE output after linoleic acid;

this would not have occurred if there was significant cross-reactivity between linoleic acid and PGE.

The linoleic acid administered in this study was virtually 100% pure (shown by GC-MS) and so the significant increase in PGE output was not due to other as yet undetermined compounds. Nor was it a non-specific effect of fat since dietary stearic acid did not affect gastric prostaglandin output.

The absence of a rise in gastric prostaglandin secretion in ulcer patients following dietary linoleic acid was unexpected. The cause of this abnormality is not explained in these experiments. It could be due to defective absorption (unlikely) or defective metabolism. Further experiments involving incubating biopsies would be necessary to define this.

d) Gastric acid output

Gastric acid output was similar in the normal subjects and duodenal ulcer. This was unexpected; it is well recognised that acid secretion tends to be increased in duodenal ulcer patients. Nevertheless, the normal subjects had no endoscopic or clinical evidence of ulcer whilst the disease group had dyspeptic symptoms and endoscopically proven ulcer. Many ulcer patients are found in clinical practice to have normal acid secretion, illustrating that gastric acid is not the only factor in

ulcer pathogenesis.

Since prostaglandins are known to suppress acid secretion it appears likely that the significant decrease in acid output observed in normal controls taking linoleic acid was mediated by prostaglandins.

The effect of linoleic upon acid secretion was clearly not merely a non-specific effect of fatty acid ingestion because it was not affected by stearic acid.

Linoleic acid had no effect upon either acid or prostaglandin secretion in ulcer subjects, suggesting that dietary manipulation of essential fatty acid intake will not modify the natural history of duodenal ulcer. This clearly questions the initial hypothesis of Hollander and Tarnawski (1986).

e) Gastric mucus output

In healthy subjects soluble mucus output was significantly increased by linoleic acid but not by stearic acid; mucus output did not increase in ulcer patients taking linoleic acid. The increase in mucus output in the healthy controls taking linoleic acid is probably due to the increase in PGE output because there was no increase in PGE output in the controls taking stearic acid nor in the duodenal ulcer patients.

This observed increase in mucus following stimulation of endogenous PGE agrees with the findings of Sellers et al

(1986) and Waterbury et al (1986) who administered prostaglandin E analogues and found an increase in the adherent mucus layer. Bolton et al (1978), Domschke et al (1978), Johansson and Kollberg (1978) all found increases in soluble mucus after administration of PGE analogues.

f) Serum gastrin concentration

The serum gastrin concentration significantly increased in normal subjects taking linoleic acid but not when taking stearic acid. Linoleic acid ingestion was not associated with changes in gastrin concentration in ulcer subjects. It is possible that the increases occurring during linoleic acid ingestion in normal subjects were secondary to suppression of gastric acid, although the magnitude of this suppression was small; certainly less than that associated with H_2 receptor antagonist therapy, which has only modest and inconsistent effects upon gastrin concentrations. The effects of orally administered prostaglandin analogues upon serum gastrin concentration are controversial. Several groups have measured serum gastrin concentrations after administration of endogenous PGE or its analogues in ulcer patients. Tytgat and Huibregste (1981) found no change in mean serum gastrin concentration in patients with acute duodenal ulcers who were treated with 15(R)-

15-methyl PGE₂ for four weeks. In contrast Mahachai et al (1984) found that there was a reduction in mean serum gastrin concentration in patients with an inactive ulcer treated with Enprostil (a de-hydro prostaglandin E₂ analogue) for one week. Konturek et al (1979) found that 15(r)-methyl PGE₂ inhibited the serum gastrin response to a meal.

CHAPTER 3

THE GASTRIC MUCOSAL PROTECTIVE PROPERTIES OF LINOLEIC ACID

1. INTRODUCTION

Experimental models of cell injury

- a) Aspirin-induced mucosal injury
- b) Alcohol-induced mucosal injury
 - i) endoscopic appearance
 - ii) histology
 - iii) nuclear proliferation
 - Autoradiography
 - Flow cytometry

2. METHODS

- a) Aspirin study
 - i) design
 - ii) labelling of red cells
- b) Alcohol study
 - i) design
 - ii) alcohol-induced injury
 - macroscopic appearance
 - microscopic changes
 - autoradiography
 - flow cytometry

3. RESULTS

- a) Aspirin-induced blood loss
- b) Alcohol-induced injury
 - i) endoscopic appearance
 - ii) histological changes
 - iii) autoradiography
 - iv) flow cytometry

4. DISCUSSION

- a) Aspirin-induced blood loss
- b) Alcohol-induced injury
- c) Linoleic acid and endogenous gastric prostaglandin secretion
- d) Comparison of flow cytometry with autoradiography

1. INTRODUCTION

In experimental animals and man, pharmacological concentrations of prostaglandins and prostaglandin analogues protect the gastric mucosa against injury by a wide variety of damaging agents. These include aspirin (Cohen et al, 1985), ethanol, sodium hydroxide and hypertonic saline (Robert et al, 1979). The mechanisms are incompletely understood although prostaglandins are known to stimulate gastric mucus and bicarbonate secretion (Rees et al, 1984), and stimulate repair mechanisms (Tytgat et al, 1982). The clinical significance of these mucosal protective actions is controversial; it is likely for example that prostaglandin analogues heal ulcers by suppressing gastric acid secretion rather than enhancing mucosal protection (Hawkey and Walt, 1986). Furthermore, although the pharmacological effects of prostaglandins are well substantiated, their physiological role in gastric function and mucosal repair is poorly understood. This is partly because it is difficult to reliably measure prostaglandins in mucosal biopsies and gastric juice (Samuelsson et al, 1975). A physiological role has been inferred from the observation of "adaptive cytoprotection" in which exposure of gastric mucosa to a low concentration of a damaging agent stimulates local prostaglandin release and protects the gastric mucosa

against subsequent exposure to a higher concentration of the same or another agent. This phenomenon is only indirect evidence of a protective role for endogenous gastric prostaglandins since the sensitizing exposure may also affect factors unrelated to prostaglandins which could then protect the stomach.

In this study an alternative approach to defining the mucosal protective effect of endogenous gastric prostaglandins has been adopted. This approach is based upon the observation that gastric prostaglandin output can be modulated by changing dietary essential fatty acid intake. Gastric prostaglandin output decreased in rats fed a diet deficient in linoleic acid (LA), whilst a linoleic acid-rich diet increased PGE output (Schepp et al, 1988). Arachidonic acid supplementation both increases gastric PGE output and protects the rodent fore stomach against ulceration by ethanol (Tarnawski et al, 1983). In the previous chapter it was shown that relatively modest dietary supplementation with linoleic acid (3g daily) increased mean gastric PGE output from 498 (\pm 110)ng/hr (SEM) to 1230 \pm (300)ng/hr. The output of 13,14 dihydro 15-keto PGE₂ (the major metabolite of PGE) increased in a similar manner and gastric acid secretion was also modestly, but significantly, depressed. Thus, by stressing the stomach of individuals before and after the administration of linoleic acid, the role of endogenous gastric prostaglandins can be determined. Using this model, it

was possible to investigate whether changes in dietary essential fatty acid intake could influence the gastroduodenal micro-environment, and alter its susceptibility to damage from non-steroidal anti-inflammatory drugs or peptic ulcer.

In the studies described in this chapter the effects of linoleic acid upon experimental gastric mucosal damage induced by aspirin and ethanol were determined.

Experimental models of cell injury

a) Aspirin-induced mucosal damage

Aspirin damages the gastro-duodenal mucosa by inhibiting mucosal protective mechanisms. It inhibits the enzyme prostaglandin synthetase (Vane , 1971) thereby disrupting the prostaglandin pathway. The exact mechanism by which aspirin causes gastric ulceration is not known, but it has been hypothesized that its ulcerogenic action is due to the withdrawal of the protective qualities of prostaglandins (Konturek et al, 1981). Aspirin can cause lesions whether given intra-gastrically or parenterally (Bugat et al, 1976; Brodie and Chase, 1967) and the severity of these lesions can be increased by gastric acid stimulation or intragastric perfusion of HCl solution (Guth et al, 1979). The likely explanation for this observation is that un-ionized aspirin diffuses from the acidic environment of the stomach through the

mucosa, accumulates within the epithelial cells because of the high pH and disturbs mitochondrial phosphorylation and cell buffer system: (Svanes et al, 1979; Spenney and Brown, 1977). Aspirin also appears to alter mucosal blood flow, causing venular constriction, capillary stasis and increased endothelial permeability.

In man, aspirin ingestion causes erythema and erosions in the gastric mucosa. This can be visualized and measured at endoscopy and blood loss can be measured in the faeces. Cohen et al (1985) have shown that the prostaglandin E₁ analogue, Misoprostol, reduces aspirin-induced bleeding.

There are two main ways of detecting and measuring blood loss from the gastro-intestinal tract. These involve measuring the peroxidase activity of iron containing compounds or labelling the red cells with a radio-active tracer. In the former group, the most commonly employed tests are the guaiac or orthotolidine tests. Both depend on the peroxidase activity of iron-containing haemoglobin derivatives, which catalyse the oxidation of guaiac or orthotolidine in the presence of hydrogen peroxide. Both are used in clinical practice and there have been many papers published based on this method. Their validity has been challenged by Van Essen et al (1985). They found them to be unsatisfactory as the effect of haemoglobin on the conversion of orthotolidine is decreased by low pH, high specific gravity and protein. The presence of

reducing substances can give false negative results and oxidising substances can give false positive results. The results are not always reproducible nor accurately quantitative.

The Cr^{51} method of determining gastro-intestinal blood loss is therefore generally regarded as more reliable (Rhys Davies 1984). It involves removing a quantity of blood from the patient, labelling the red cells with radioactive Cr^{51} , re-injecting the labelled red cells into the patient and measuring faecal radio-activity. It is a well-tried method and provides quantitative and reliable data.

Cr^{51} is a widely used radio-nucleotide in both clinical practice (red-cell survival studies and protein-losing enteropathies) and in medical research. It binds to the β chain of adult haemoglobin (HbA). On average 95% of red blood cells are labelled with Cr^{51} . Cr^{51} has a half-life of 27.8 days and emits gamma-radiation which can be easily measured. The mean total radiation exposure in each patient was approximately 2.4 MBq.

b) Alcohol-induced mucosal damage

Alcohol damages the gastric mucosa by several mechanisms (Oates and Hakkinen, 1988). Concentrated alcohol causes necrosis of superficial mucosal cells by precipitating cytoplasmic constituents. In response, mucosal mast cells release the vasoactive mediators

leukotriene C₄ and histamine, which cause constriction of mucosal venules followed by dilatation of arterioles, venous engorgement, increased capillary pressure, exudation of fluid, haemolysis and subsequent basal thrombosis, stagnation and cellular death.

In the rat fore-stomach, absolute ethanol causes deep ulceration which can be prevented by pre-treatment with prostaglandins, arachidonic acid or linoleic acid. Hollander and Tarnawski (1985) have shown that superficial necrosis occurs whether or not animals have received PGE but in animals receiving PGE cells deep within the proliferative pits of the gastric gland are protected and re-epithelialisation is enhanced.

In man, alcohol-induced mucosal ulceration can also be used as an experimental model of mucosal injury. In this study, damage was assessed in several ways.

- i) The endoscopic appearances following alcohol-induced damage were assessed using a visual scoring system modified from Agrawal et al (1987). The problem with this approach is that it is subjective; the endoscopist cannot be blinded as to whether or not linoleic acid had been given because the remnants of the capsule or green fluid can be seen.
- ii) The endoscopic biopsies were taken adjacent to the damaged area. These were objectively assessed by an independent histologist who received the biopsies

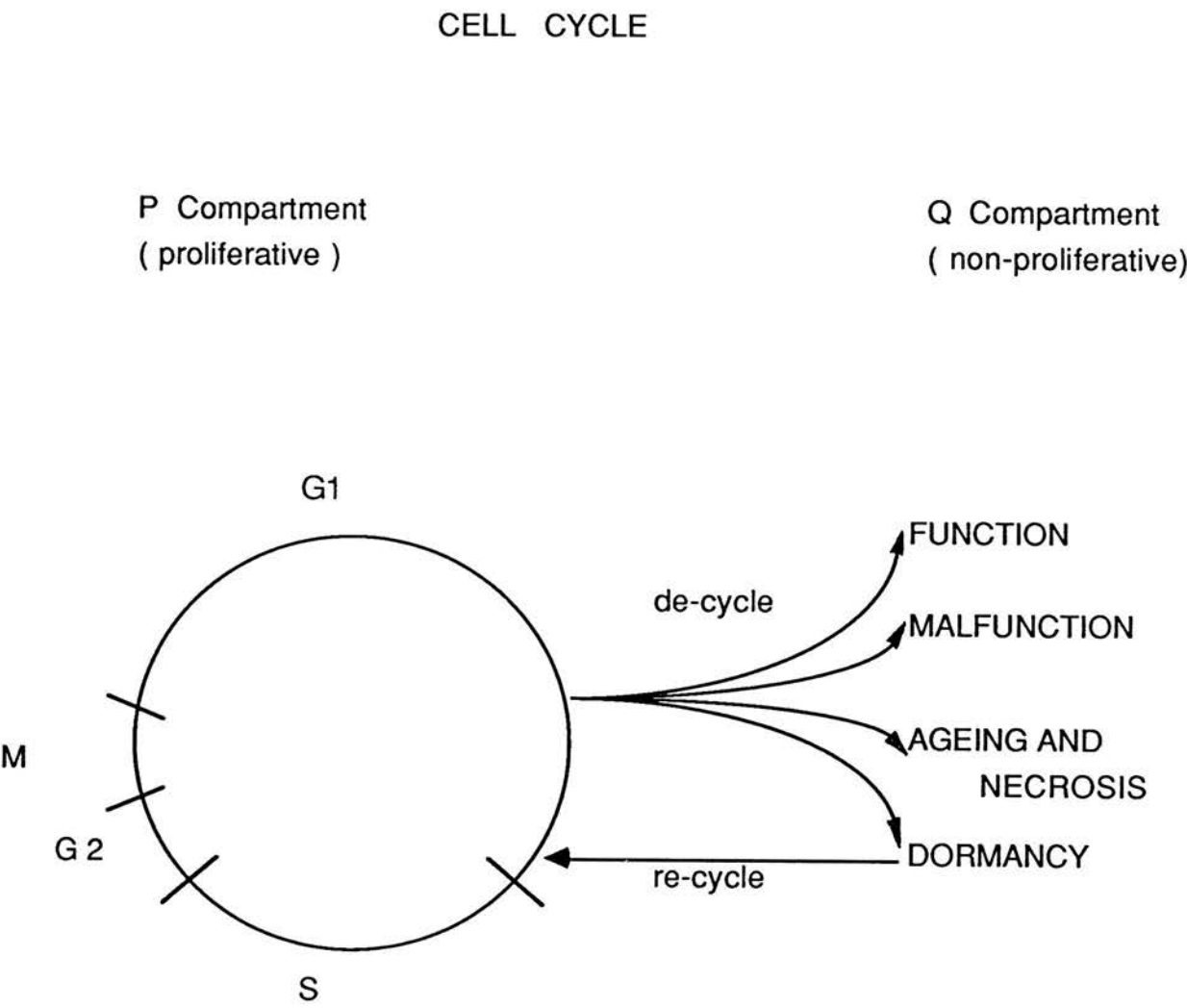
in a blinded, random order.

iii) The rate of nuclear proliferation in cells adjacent to the damaged area was used as an index of cell viability. This approach has also been used by Hart Hansen et al (1975) and Boyes et al (1971) in other studies.

In order to assess nuclear proliferation, the proliferative area of the gastric pits was studied. In the antrum these progenitor cells are located in the isthmus of the gland (Leblond et al, 1948), from which they divide and migrate to the surface. Epithelial renewal occurs every 3 - 5 days but when the epithelium is damaged, restitution occurs within a matter of a few hours.

During the cellular repair process, the progenitor cells synthesise proteins prior to cell division (mitosis). These cells can be identified (and counted) in either the synthetic "S" phase or during mitosis itself. The process of cell division can be divided into four phases - G1, S, G2, M (Howard and Pelc, 1953). Cairnie et al (1965) divided renewing tissue into two compartments: the "P" compartment containing cells actively engaged in the cell cycle and the "Q" compartment containing non-proliferating cells (Fig 3:1).

fig 3:1



From Aherne et al (1977)

- "G1" is a variable period when the cell is actively functioning; (Mueller, 1971; Aherne et al, 1977), cells may enter the proliferating cell cycle or move to the "Q" compartment and become dormant.
- In the "S" phase DNA replication occurs.
- "G2" is the brief phase when the cell prepares for the initiation of mitosis.
- The "M" phase represents mitosis in which identical genetic material is apportioned to daughter cells (Tobey et al, 1971).

For the purposes of this experiment the S phase was studied. Two methods were used, autoradiography (ARG) and flow cytometry.

Autoradiography (ARG)

DNA and RNA have three bases in common - adenine, guanine and cytosine. Only DNA has thymine and only RNA has uracil. By using tritiated thymidine (^3H -TdR) as a label, DNA metabolism can therefore be followed. In essence this involves incubating mucosal biopsies with ^3H -TdR followed by autoradiography and counting the number of labelled nuclei.

^3H -TdR incorporation is used to determine the proportion of cells in the cell cycle which are in the "S" phase. In the context of this study it can also be used to

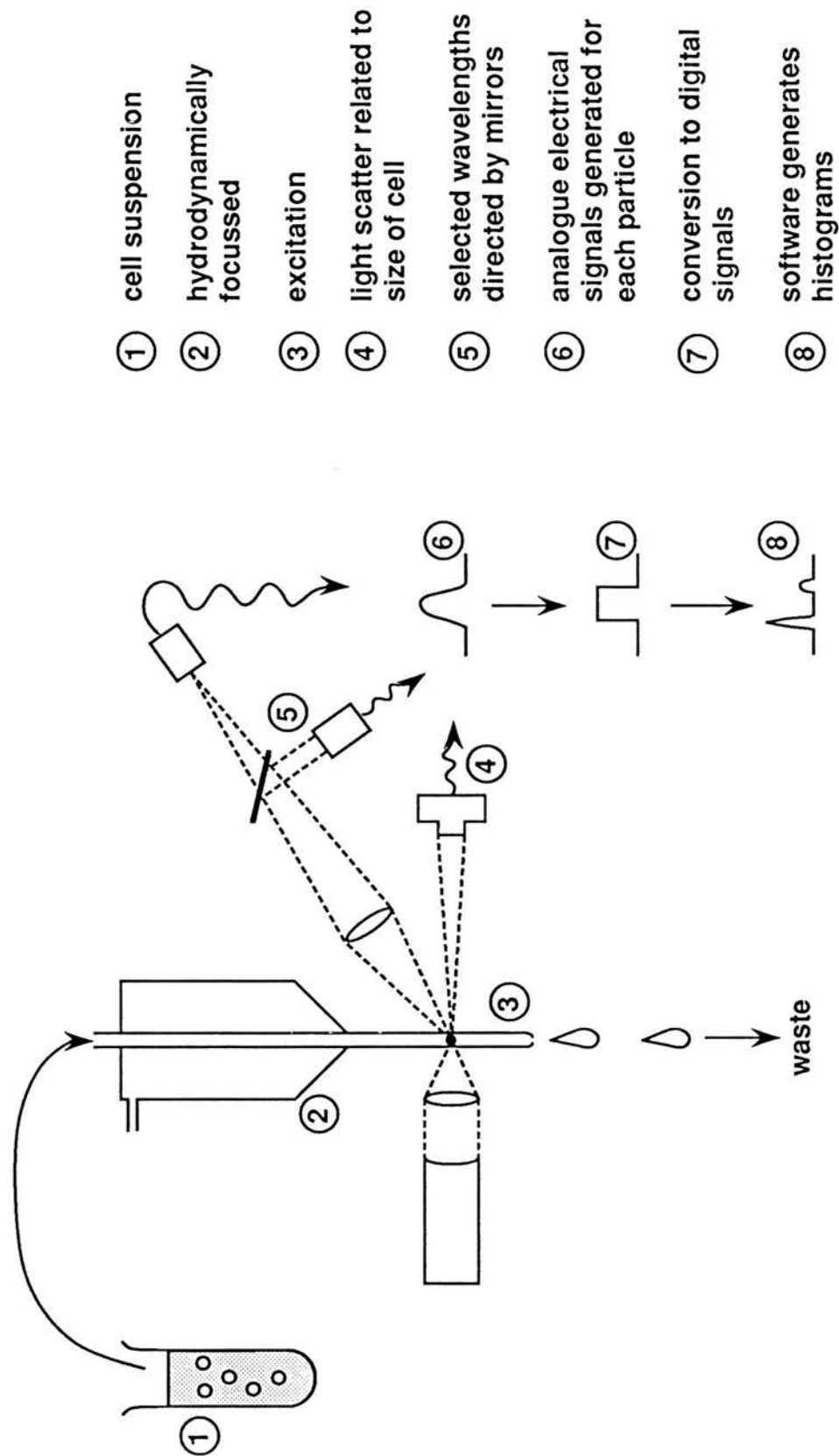
indicate of the number of surviving cells.

Flow Cytometry

This is based on the stoichiometric reaction of fluorescent dyes with DNA. Cells in the G0/G1 phase have a diploid amount of DNA, cells undergoing mitosis have a double diploid amount and those in "S" phase have an amount in between. If cells contain an abnormal amount of DNA (aneuploid) they can also be detected.

Flow cytometry was originally used to assess cell numbers and size. It was subsequently developed by Kametsky et al (1965) to quantify nucleic acid concentration using the absorption and scatter of light. In 1969 Mullaney et al produced the first DNA histogram with clearly defined G0/G1, S, G2 and M phases. This has had considerable impact on the study of cellular events. Its main advantage is that it enables the quantification of a number of parameters on a large number of cells giving rapid and reproducible results. Flow cytometry has been little studied in the gastric mucosa. There are methodological problems; for example the method depends upon the preparation of a single cell suspension - this is simple for lymphoid tissue but in the case of the stomach it necessitates grinding and enzymatic degradation of the tissue. The DNA is then stained, filtered to remove clumps and syringed to break up small aggregations. The principle of the flow cytometer is illustrated in Fig 3:2.

Fig 3:2 Schematic diagram of flow cytometer

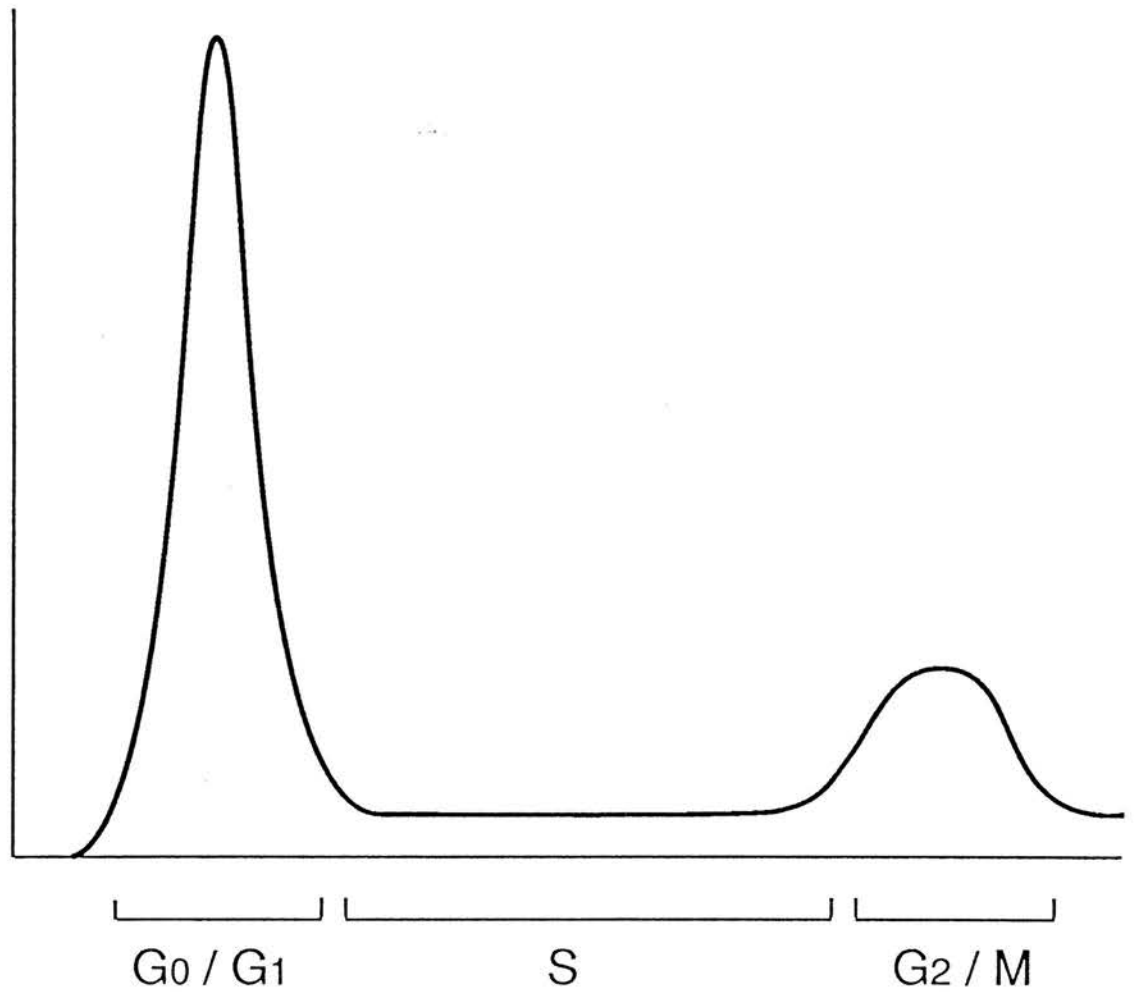


The stained cell suspension is injected into the flow cell of the flow cytometer (1) where it is hydrodynamically focussed (2) to pass through the interrogation point, excitation taking place in either an enclosed quartz flow cell or in air (3). The cell interacts with the laser light (emitted at 488 nm) scattering it in all directions; light scattered in a forward direction is related to the size of the cell, and that at 90 degrees to the amount of refraction of its internal structure (4). Volume and polarization measurements are also possible. Analogue electrical signals are generated for each particle (6) and converted into digital signals (7) for processing by the computer software (8) to generate parameter correlated histograms. Many stains are available (Shapiro et al, 1986). Most DNA stains will not enter a viable cell and alcohol fixation or detergent treatment is necessary to render the plasma membrane permeable (Traganos et al, 1977). In this study propidium iodide was used: when excited at 488 nm it emits light in the red spectrum at 580 nm. The intensity of the red light emitted by the excited nuclei correlates to the amount of DNA in the nucleus. The red light is sensed by a photomultiplier tube which amplifies the signal prior to converting it to a digital pulse. The accumulated pulses from a sample are then used to generate a histogram (Fig 3:3).

A variety of mathematical methods have been developed for subdividing DNA histograms into their cell cycle

Fig 3:3

Schematic diagram of DNA histogram from flow cytometer



components - G0/G1, S, G2/M. All models are held to be relatively accurate although differences have been observed. In this study the PARA 1 software was used: the method assumes a Gaussian distribution of the G0/G1 and G2/M phases and then fits a rectangular model for the "S" phase. By subtraction of the "S" phase from the Gaussian distribution, the percentage of events in each phase can be derived. It assumes that the cells are in asynchronous growth.

2. METHODS

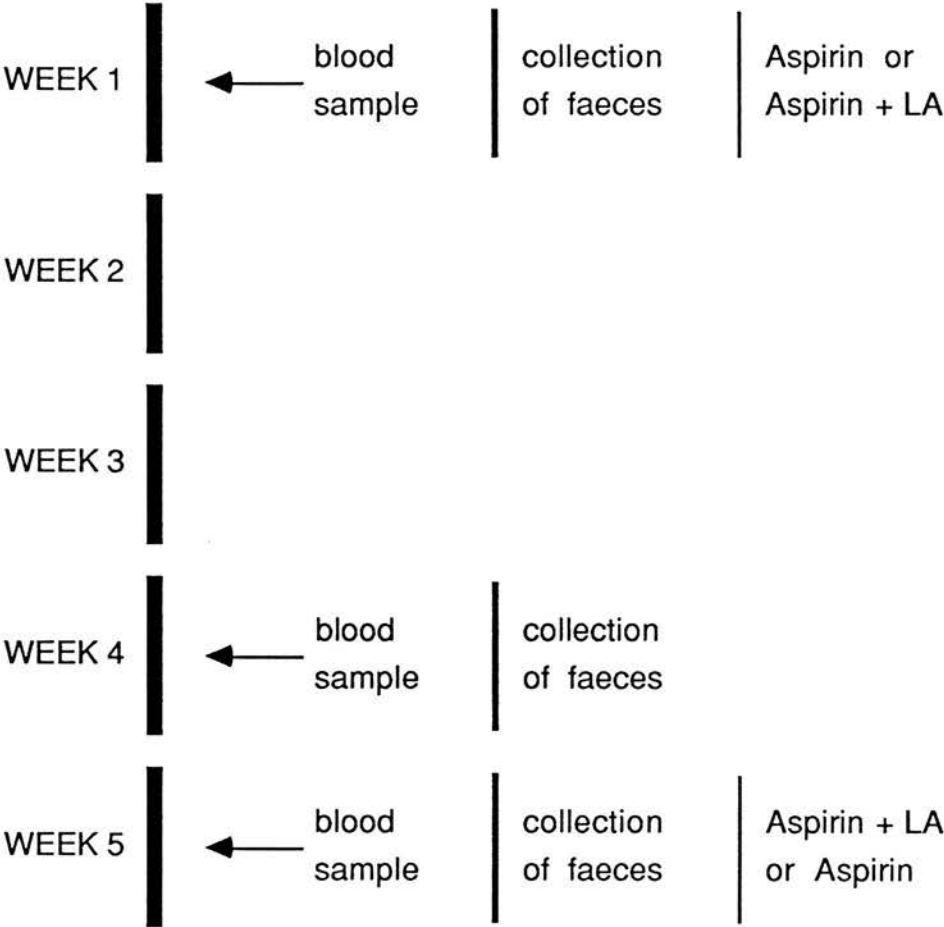
a) Aspirin study

i) Design

Aspirin-associated gastro-intestinal blood loss was measured in 10 healthy volunteers aged 22 to 40 years (mean 31.5 years): none had a history of dyspepsia, smoking nor took any medications. A 15 ml venous blood sample was obtained from each subject, the red cells were labelled with 2.4 MBq of Cr⁵¹ and then reinjected into a vein. The study lasted 5 weeks (fig 3:4), aspirin being taken during week 1 and week 5. Five subjects were randomised to aspirin alone on week 1 and the LA/aspirin combination on week 5; the other 5 received LA/aspirin in week 1 and aspirin alone during week 5. 1.2g of soluble aspirin was taken 8 hourly on an empty stomach.

fig 3:4

OUTLINE OF PROTOCOL OF ASPIRIN - INDUCED
BLOOD LOSS STUDY



1g of linoleic acid was taken 30 minutes prior to the aspirin during the combination weeks. All faeces were collected during week 1 and week 5, separated by a three week "washout" period. Radioactivity was measured using a gamma counter and the amount of blood loss was calculated by reference to the radioactivity of a 10ml, mid-week sample of venous blood. Linoleic acid was administered in gelatine-coated capsules containing the free acid (rather than ester), suspended as a micellar solution in the non-ionic detergent Pluronic F68.

(ii) Red cell labelling

15 mls of blood were withdrawn and placed in a sterile universal container with tri-sodium citrate as an anti-coagulant and centrifuged at 3000 rpm for 10 minutes. The plasma and buffy coat were removed (to remove the leucocytes to which Cr^{51} can also bind). 2.4 MBq of Cr^{51} were added to the red cells in the form of sodium chromate (12 MBq in 10ml) and incubated at 37 °C for 10 minutes. The blood was washed three times with sterile saline after centrifuging (to remove any of the unbound isotope); it was then made up to 15mls with sterile saline and returned to the patient.

The radioactivity in the blood samples was measured using an LKB automatic γ - counter. All stool samples were counted in a large (Part body) sample γ -counter. As different machines were used for counting, a correction factor was derived by comparing 0.2 MBq of Cr^{51} in 200mls

of chromic chloride: 200mls aliquots were measured in the large sample counter and 5mls aliquots in the sample counter.

For experimental blood samples 5mls were taken and counted in the sample counter for 400 seconds, an empty tube was also counted to measure background radioactivity.

Stool samples were collected in wax cartons and counted for 400 seconds in the part body counter. Background counts were also measured.

b) Alcohol study

i) Design

The damaging effects of ethanol upon the gastric mucosa were assessed in 6 volunteers aged 22 to 38 (mean 26.6) years. After an overnight fast each subject underwent routine upper gastrointestinal endoscopy using an Olympus GIF P10 gastroscope. Intravenous sedation was not administered although the pharynx was anaesthetised using Lignocaine spray. Normality of the oesophagus, stomach and duodenum was confirmed endoscopically and two biopsies were taken from the gastric antrum. 30 mls of 80% ethanol were then directly infused onto an accessible, convenient part of the gastric antrum using a catheter passed through the biopsy channel of the endoscope. The endoscopic appearances were carefully

documented 3 and 10 minutes after ethanol infusion and two biopsies were taken at both time points from the mucosa around the damaged area. The endoscope was removed, then reintroduced 30 and 90 minutes after ethanol for further visual inspection and biopsies.

Each subject was studied on two occasions separated by two weeks. On one occasion subjects were pre-treated with 48 hours of linoleic acid, 1g 8 hourly, and the experiment was conducted exactly one hour after ingesting the last capsule of linoleic acid, whereas on the other occasion they had no pre-treatment. The subjects were crossed-over and randomised.

ii) Alcohol-induced mucosal injury was assessed in the following way :-

Macroscopic appearances were graded using a method modified from Agrawal et al (1986)

- 0 = normal
- 1 = marked diffuse hyperaemia
- 2 = single haemorrhage < 2 mm
- 3 = 2 - 5 haemorrhages < 2 mm
- 4 = numerous or confluent lesions

Microscopic changes were assessed using several techniques - biopsies were processed using standard histological techniques and stained using haematoxylin and eosin. The degree of damage was assessed by an independent histologist (Dr. Alastair Lessels) who had no

knowledge of the mucosal appearances or timing of the biopsies. The presence or absence of superficial erosions and cell necrosis was determined for each section.

Autoradiography was performed on other biopsies using a modification of the method described by Hart Hansen et al (1975). Immediately after the biopsy was taken it was placed in 2mls of RPMI 1640 cell culture medium (Flow Laboratories, Irvine, Scotland) at 37° for one minute. The mixture was gently agitated to remove debris and mucus. The biopsy was transferred to a fresh solution of RPMI containing 20ug/ml of ^3H -TdR (Amersham International) at pH 7.4 and incubated for one hour at 37°C. The tritiated medium was poured away and the biopsy washed in unlabelled RPMI for 10 minutes then fixed in 10% buffered formal saline for 24 hours at room temperature.

The biopsies were then processed for standard microscopy using an automatic tissue processor (Histokinette, British American Optical Co. Ltd.) which passes the sample through graded alcohols.

Biopsies were embedded in paraffin wax at 50°C, 3µm sections were cut using a Jung rotary microtome, placed onto Poly-L-lysine-coated slides and dried on a hot plate at 60°C. From each biopsy at least 6 levels were cut in both a transverse and a tangential plane.

Photographic emulsion (Ilford K5, Ilford, Essex) was applied in a dark room. It was mixed with an equal

amount of glycerine (to dilute and reduce background fog) and mixed in a water bath at 43°C. The slides were carefully dipped to ensure even coating, placed in a dessicator overnight and stored for 4 weeks in a dark enviroment at 4°C.

The slides were developed by placing them in Kodak D19b developer for 3.5 minutes followed by washing in water and then placing in Kodak Unifix at 35°C for 10 minutes. They were finally washed in tap water for 15 minutes and then stained with haematoxylin and eosin in a routine manner. The silver grains due to ^3H -TdR autoradiography were easily counted by light microscopy (fig 3:5). Cells were considered to be labelled if there were five or more grains overlying the nucleus. About 40% of slides were unsuitable for counting for technical reasons: these included fogging of the slide during development, poor sectioning or poor orientation. Glands were only counted if there was at least one labelled cell in the cross-section, indicating that it was the proliferative zone. This inevitably meant that there was an over-estimation of the labelling index (as glands without labelled cells have not been counted). This can be corrected for by a formula devised by Hart Hansen et al (1975) which is depicted in Fig 3:6.

Background counts of silver grains were estimated by counting the number of grains over a lymphocyte or the number of grains over a mitotic figure.

Cells were counted using a square-grid eyepiece fitted to

Fig 3:5

AUTORADIOGRAPHY (ARG)
TANGENTIAL SECTIONS THROUGH THE GASTRIC MUCOSA

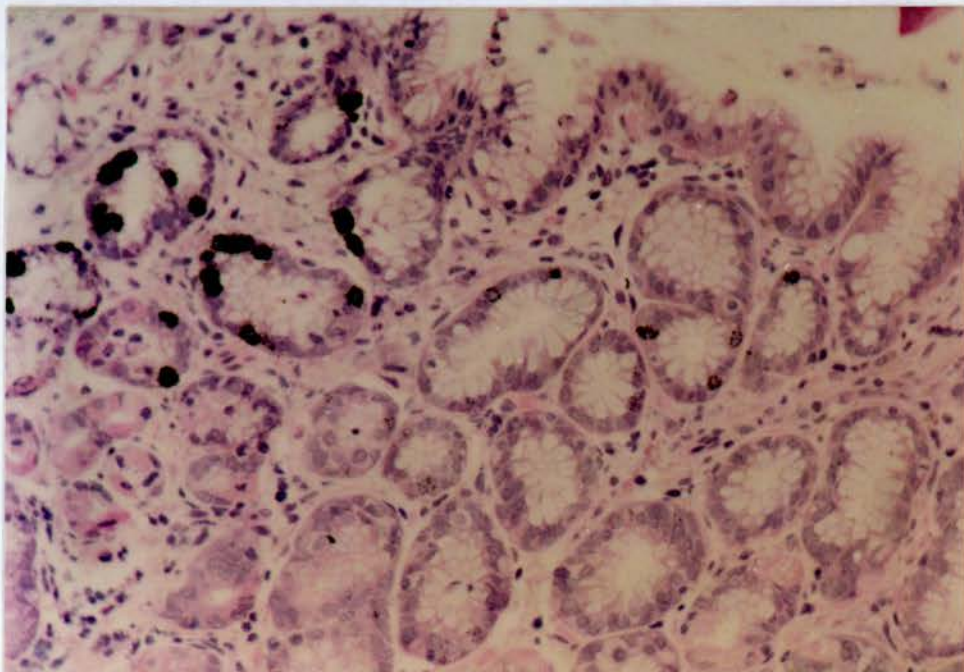
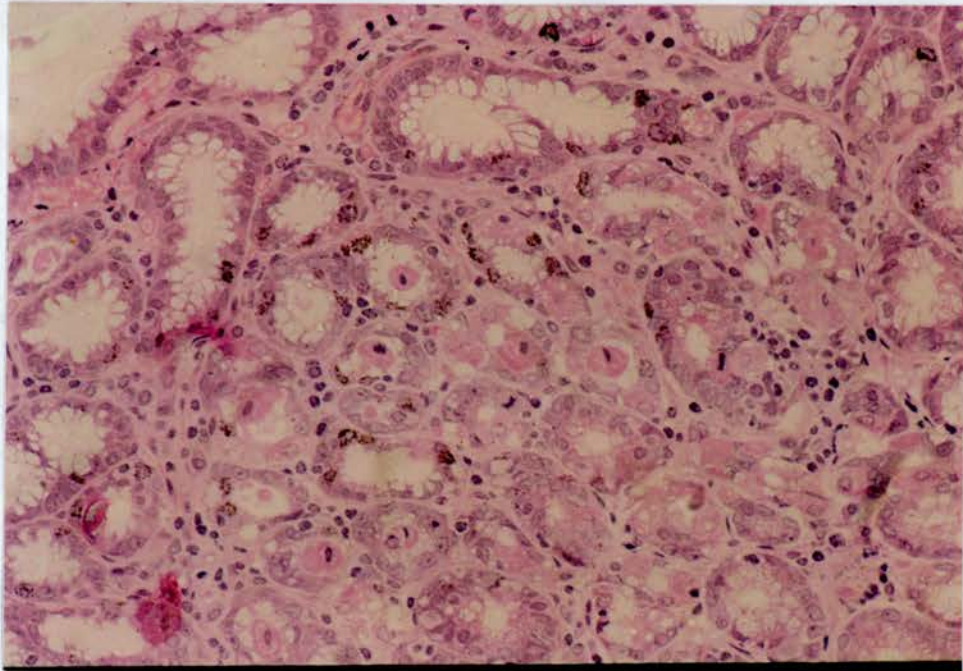
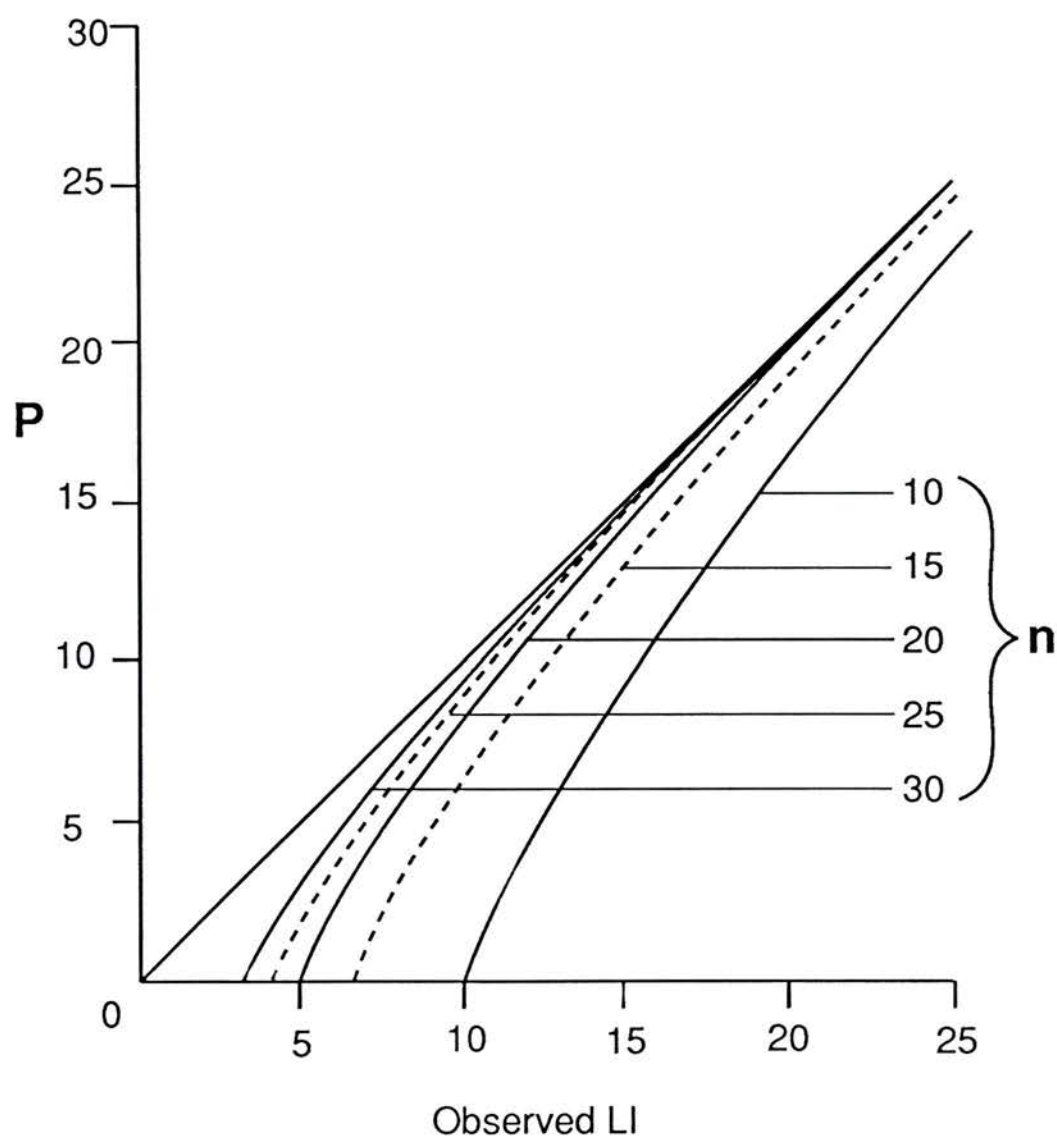


Fig 3:6



Nomogram for correction of labelling indices estimated on basis of cell counts in cross sections of foveolae. **p** represents the corrected labelling index, **n** the mean number of cells in the cross sections, and **L.I.** is the actual estimated labelling index.

*After Hart Hanson O. et al
Gut.1975; 16: 23-27*

a Leitz Wetzlar Ortholux II microscope calibrated to give numbers of cell/mm². On transverse sections (fig. 3:7) at least 40 eyepiece grids were counted using a 400 magnification and the mean number of labelled cells was obtained. The frames were orientated with the surface and included isthmus and neck of the gland.

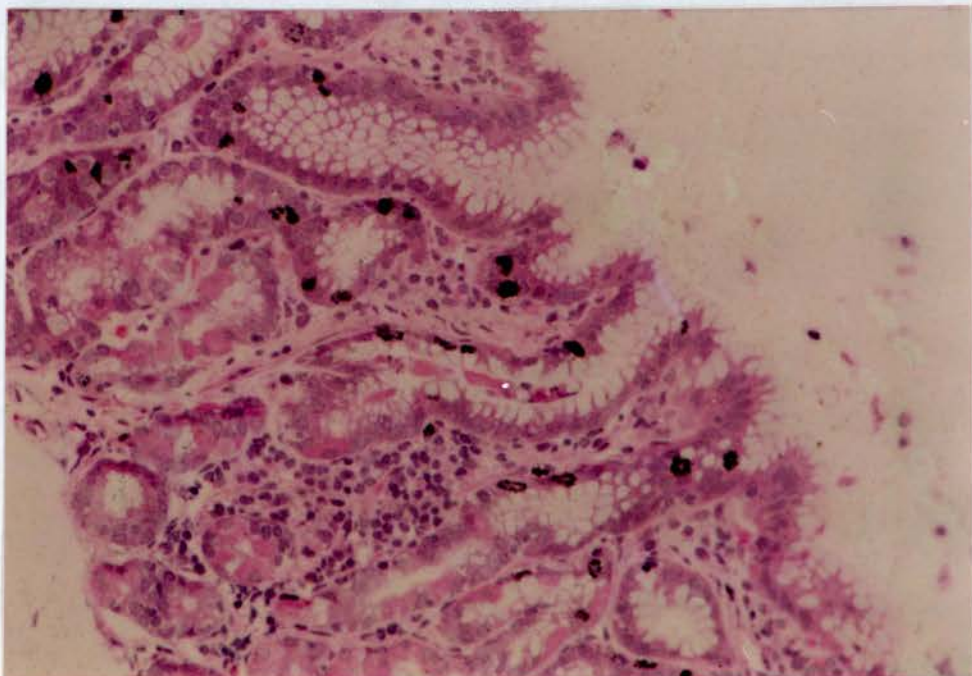
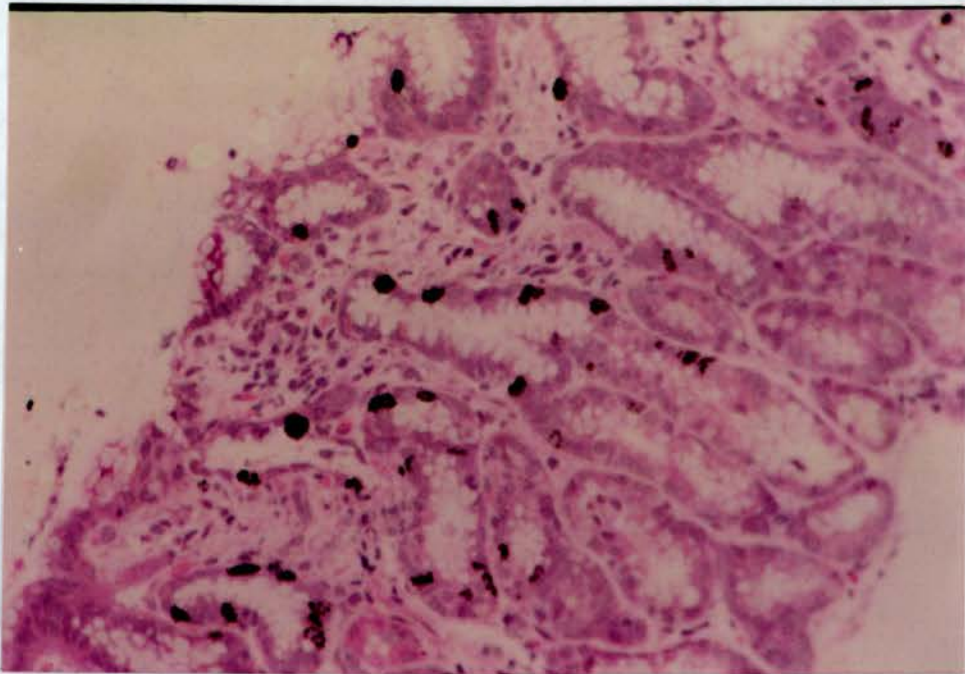
Accuracy was assessed by counting one slide on 10 different occasions over a 3 month period. This was to ensure that counting technique and reproducibility were maintained for the duration of the study. The labelling index of this slide ranged from 14.9 - 17.0%. The mean was 16.4% (SEM 0.20). The relative standard deviation was 6.5%.

Flow cytometry was performed on other specimens taken from the gastric mucosa. A biopsy was fixed in 98% methanol for 24 - 72 hours and then ground over wire mesh to disaggregate the tissue. It was then placed in a solution of 0.05% porcine pepsin in N Saline (which was adjusted to pH 1.5 using 2N HCl) and allowed to react for 30 minutes at 37°C. It was vortexed at 10 minute intervals, washed twice in phosphate-buffered saline containing 1 % bovine albumin (to halt the pepsin). This solution was filtered through washed nylon wool, and re-suspended in staining solution (containing propidium iodide 50ug/ml, 0.1M hypotonic TRIS, 5mmol MgCl, 1mg/ml ribonuclease). Counting was performed using a Coulter Flow Cytometer (Epics type CS, Hialeh, Florida), by Mr G

Fig 3:7

AUTORADIOGRAPHY (ARG)

LONGITUDINAL SECTIONS THROUGH THE GASTRIC MUCOSA



3. RESULTS

a) Aspirin-induced blood loss

All tolerated the large dose of aspirin well although most had epigastric discomfort initially. Faecal blood loss was significantly elevated in all volunteers while taking aspirin and fell during the "washout" period. Mean blood loss was 36.6 (\pm 5.8) mls/week when aspirin alone was taken (tab 3:1). It was almost identical at 34.7 (\pm 6.5) mls/week in the week when aspirin and linoleic acid were taken together (fig 3:8).

b) Alcohol-induced injury

All the subjects tolerated these experiments remarkably well. Repeated endoscopies were easily accomplished and alcohol infusion caused no symptoms.

i) Endoscopic appearances

Alcohol infusion was almost immediately followed by erythema and mucosal oedema. By 90 minutes petechial haemorrhages and superficial erosions were seen (fig 3:9). The endoscopic appearances were similar whether or not the subjects received linoleic acid prior to the endoscopy (tab 3:2).

table 3 :1

FAECAL BLOOD LOSS (ml / week) CAUSED BY ASPIRIN
BEFORE AND AFTER LINOLEIC ACID

VOLUNTEER	ASPIRIN ALONE	ASPIRIN \pm LA
1	44.3	37.1
2	29.7	23.4
3	33.9	38.6
4	29.5	22.2
5	23.9	37.4
6	30.5	51.1
7	46.5	82.5
8	72.1	25.1
9	51.7	20.0
10	4.5	9.7
\bar{x}	36.6	34.7

table 3:2

VISUAL SCORING OF DAMAGE TO THE GASTRIC
MUCOSA CAUSED BY ALCOHOL

VOLUNTEER	ALCOHOL ALONE	ALCOHOL \pm LA
1	2	1
2	2	2
3	2	1
4	2	2
5	2	2
6	2	1

Fig. 3:8

ASPIRIN INDUCED INJURY - RESULTS

Mean Faecal Blood Loss with Aspirin alone
and Aspirin with Linoleic Acid

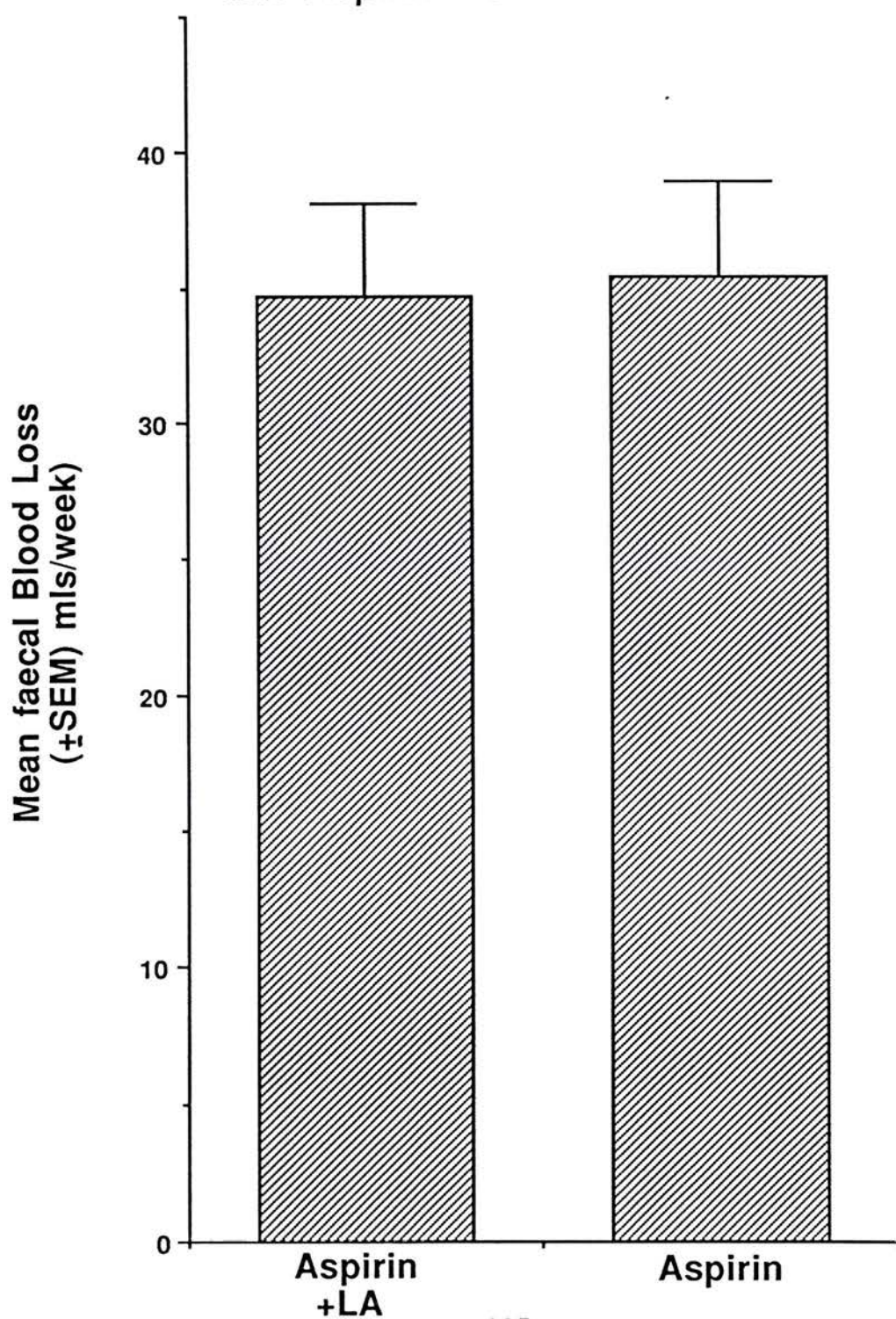
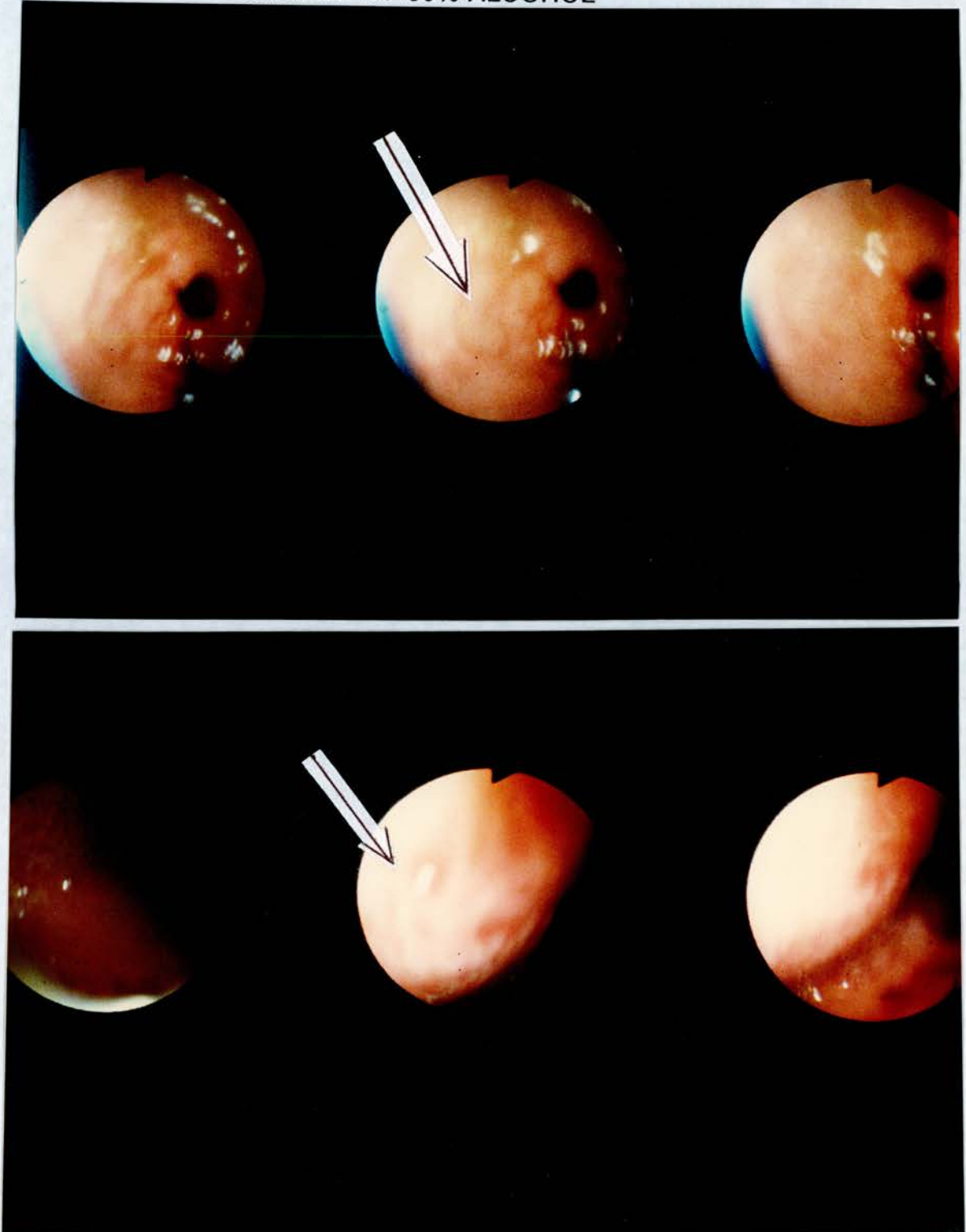


Fig 3:9

MACROSCOPIC DAMAGE TO THE GASTRIC MUCOSA
CAUSED BY 80% ALCOHOL



ii) Histological changes

The effect of alcohol on the mucosa was assessed in 2 ways histologically:- a) damage to the epithelium and b) cellular necrosis. The biopsies were all small (because many were taken and a paediatric endoscope was used) so only gross changes could be accurately measured. After the alcohol, the surface epithelium was scored as either intact (I) or damaged (D). Cellular necrosis was graded as:-

0 = no necrosis

1 = necrosis at edge, or superficial necrosis

2 = generalised or deep necrosis.

Results are shown on Tab 3:3 and Tab 3:4. Some of the biopsies showed no apparent damage to the epithelium after the alcohol, although visible damage had indeed been caused. This is probably due to the fact that the biopsies were taken from the edge of the lesion and may in some cases have missed the damaged area. Examples of the range of mucosal damage are shown in fig 3:10.

Similarly, cellular necrosis was not always seen, probably for the same reason.

There was no significant difference in terms of pathological damage following alcohol before or after the administration of linoleic acid ($p > 0.05$).

iii) Autoradiography

The labelling indices were measured in tangential and transverse sections of the biopsies. The tangential

table 3:3

PATHOLOGICAL GRADING OF MUCOSAL INJURY
AFTER ALCOHOL ADMINISTRATION , BEFORE
AND AFTER LINOLEIC ACID

EFFECT ON SURFACE EPITHELIUM

VOLUNTEER	BEFORE LA				
	0	3	10	30	90
1	I	I	I	D	---
2	I	I	I	D	I
3	D	D	I	D	I
4	I	I	D	I	I
5	I	I	D	I	I
6	I	D	I	D	I

VOLUNTEER	AFTER LA				
	0	3	10	30	90
1	I	I	I	I	---
2	D	I	D	D	I
3	I	I	I	D	I
4	I	D	I	D	D
5	D	I	I	I	I
6	I	D	I	I	I

I = INTACT
D = DAMAGED

table 3:4

PATHOLOGICAL GRADING OF MUCOSAL INJURY
AFTER ALCOHOL ADMINISTRATION , BEFORE
AND AFTER LINOLEIC ACID

EFFECT ON CELL NECROSIS

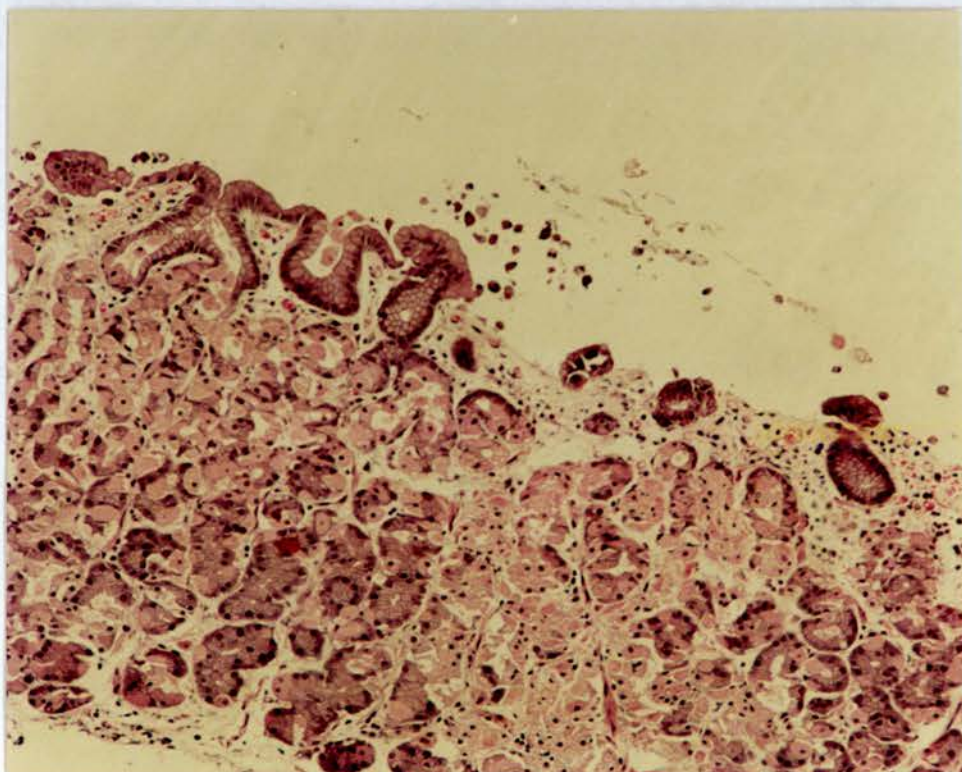
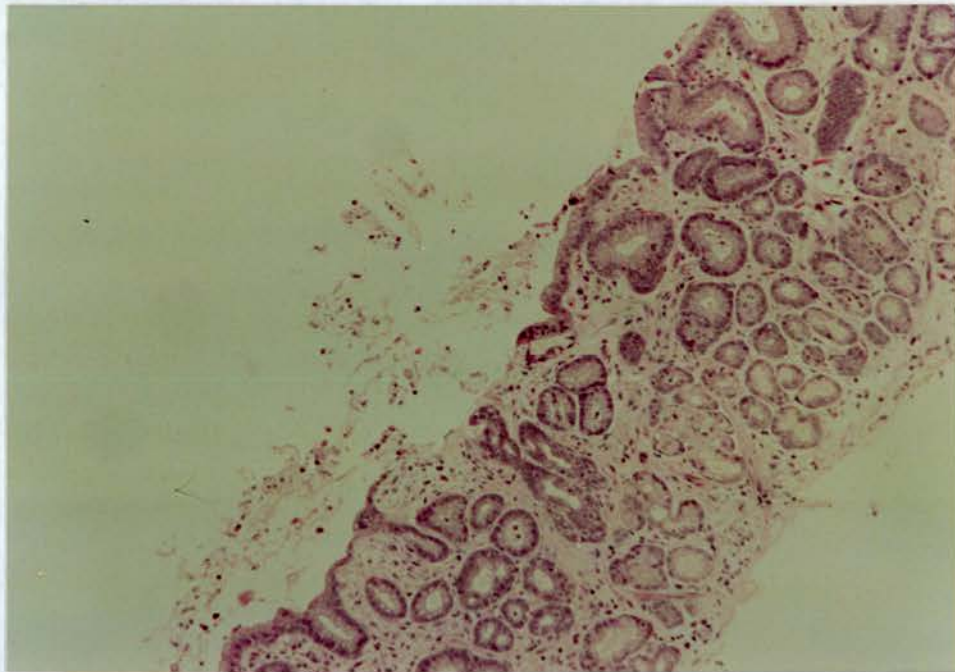
VOLUNTEER	BEFORE LA				
	0	3	10	30	90
1	1	0	1	1	---
2	1	1	1	1	2
3	1	1	0	1	0
4	1	1	1	1	1
5	0	0	1	2	1
6	0	1	1	0	1

VOLUNTEER	AFTER LA				
	0	3	10	30	90
1	0	1	0	0	---
2	2	1	2	1	1
3	0	0	1	1	1
4	2	1	0	1	1
5	1	2	1	0	1
6	1	1	1	1	0

0 = no necrosis
1 = necrosis at edge or superficial
2 = generalised or deep necrosis

Fig 3:10

HISTOLOGICAL SECTIONS THROUGH THE GASTRIC
MUCOSA SHOWING PROGRESSIVE MUCOSAL
DAMAGE CAUSED BY 80% ALCOHOL



sectioned results are shown in table 3:5 and Fig 3:11; the transverse-sectioned results are shown in table 3:6 and Fig 3:12. Following alcohol infusion there was a significant fall in the mean labelling index ($p < 0.05$) which gradually returned to the basal values over the 90-minute period. Results were very similar irrespective of linoleic acid pre-treatment.

iv) Flow cytometry

The results are presented in table 3:7 and fig 3:13. The mean values are similar to those obtained from autoradiography. The mean % of cells in "S" phase (determined by flow cytometry) did not change significantly after ethanol, and results were similar whether or not linoleic acid was administered.

Reproducibility of the histograms with 2×10^4 cells was excellent. The coefficient of variation range from 2.49-3.96 (mean 3.23).

4. DISCUSSION

a) Aspirin-induced blood loss

The main methodological criticisms of using Cr^{51} are that it involves radio-labelling of a patient's blood, the collection of all their stools and there is no way of determining where the bleeding is coming from within the gastro-intestinal tract (Hunt and Frantz, 1981). However

table 3:5

RESULTS FROM AUTOGRAPHY --- LABELLING INDEX
(as %) IN TANGENTIAL SECTION OF SLIDE

VOLUNTEER		TIME FROM ADMINISTRATION OF ALCOHOL (min)				
		0	3	10	30	90
1	pre LA	18.2	19.7	18.2	15.7	---
	post LA	18.3	15.6	18.9	18.7	---
2	pre LA	14.7	16.5	16.3	10.3	19.9
	post LA	15.4	15.7	12.8	12.1	19.5
3	pre LA	18.4	14.8	15.4	12.9	18.3
	post LA	20.5	22.7	20.1	17.8	17.0
4	pre LA	16.5	16.5	15.1	14.7	15.6
	post LA	19.7	19.4	16.7	16.8	18.8
5	pre LA	19.8	12.9	14.2	15.5	19.1
	post LA	17.7	15.3	19.1	15.3	16.3
6	pre LA	18.6	16.8	14.6	13.4	17.1
	post LA	13.4	10.3	10.3	13.0	13.1
mean	pre LA	17.7	16.2	15.6	13.7	18.0
	post LA	17.5	16.5	16.1	14.7	16.9

Fig 3:11

Autoradiography
Labelling Index (LI) of gastric mucosa after alcohol infusion.
Before and after supplementation with linoleic acid (LA)

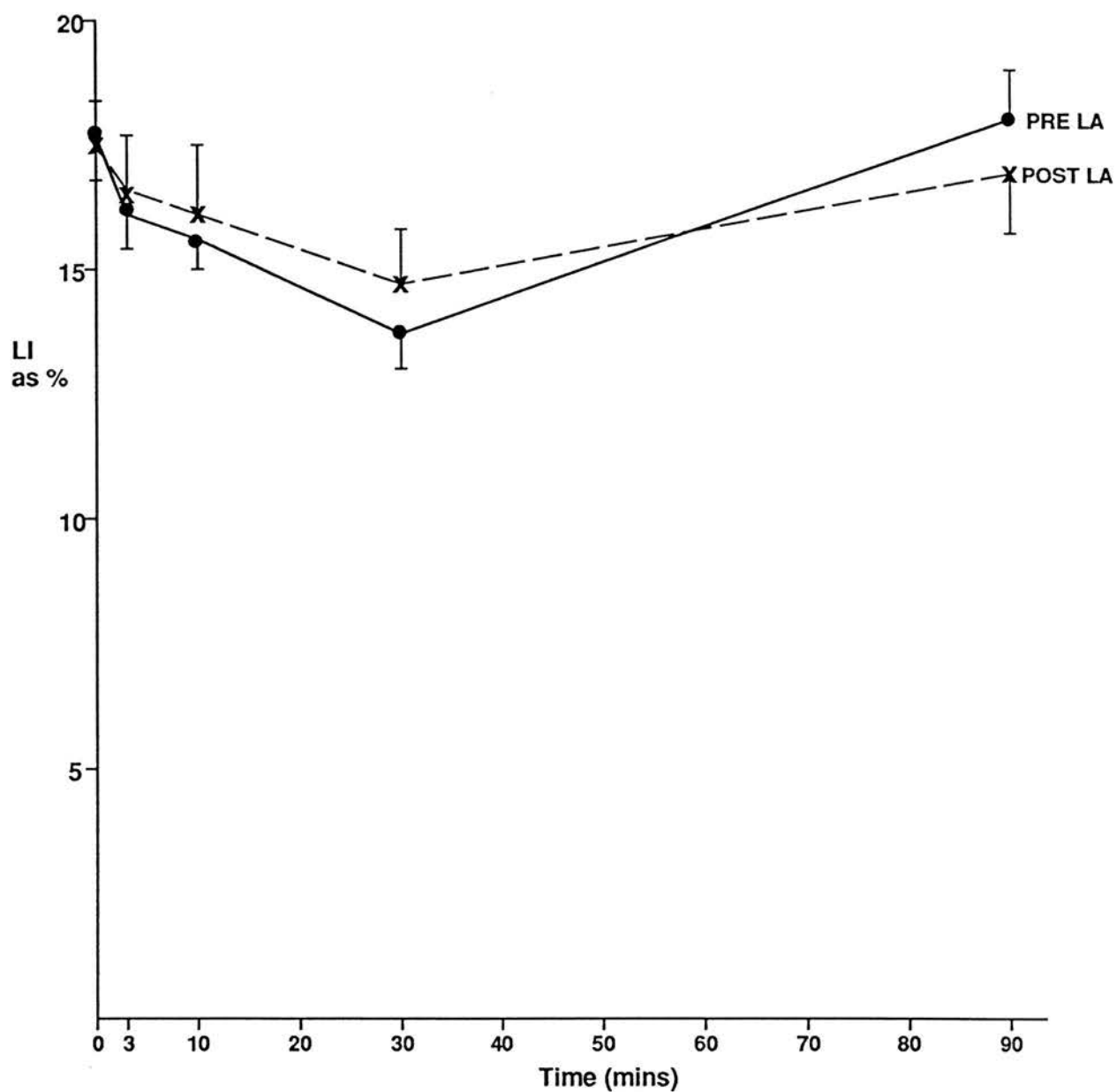


table 3:6

RESULTS FROM AUTOGRAPHY --- LABELLING INDEX
(as cells / mm²) IN TRANSVERSE SECTION OF SLIDE

VOLUNTEER		TIME FROM ADMINISTRATION OF ALCOHOL (min)				
		0	3	10	30	90
1	pre LA	104	257	191	195	---
	post LA	236	139	129	149	---
2	pre LA	181	224	198	183	299
	post LA	87	124	110	81	174
3	pre LA	272	416	122	172	263
	post LA	341	366	329	292	486
4	pre LA	197	152	136	121	102
	post LA	261	284	267	86	363
5	pre LA	288	61	94	77	146
	post LA	351	260	314	347	311
6	pre LA	164	266	121	174	113
	post LA	82	91	53	74	74
mean	pre LA	201	229	113	153	184
	post LA	226	210	200	171	281

Fig 3:12

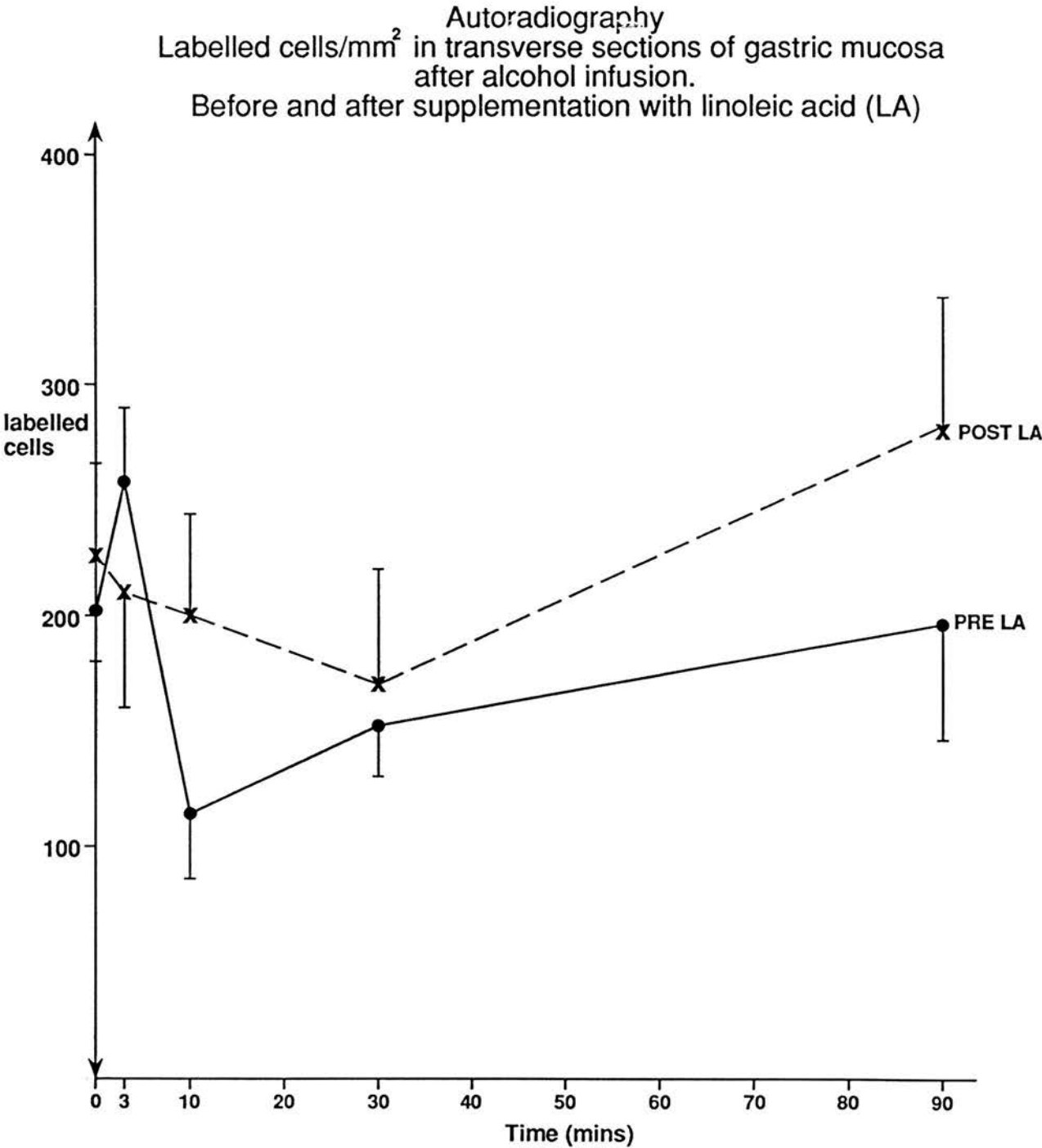
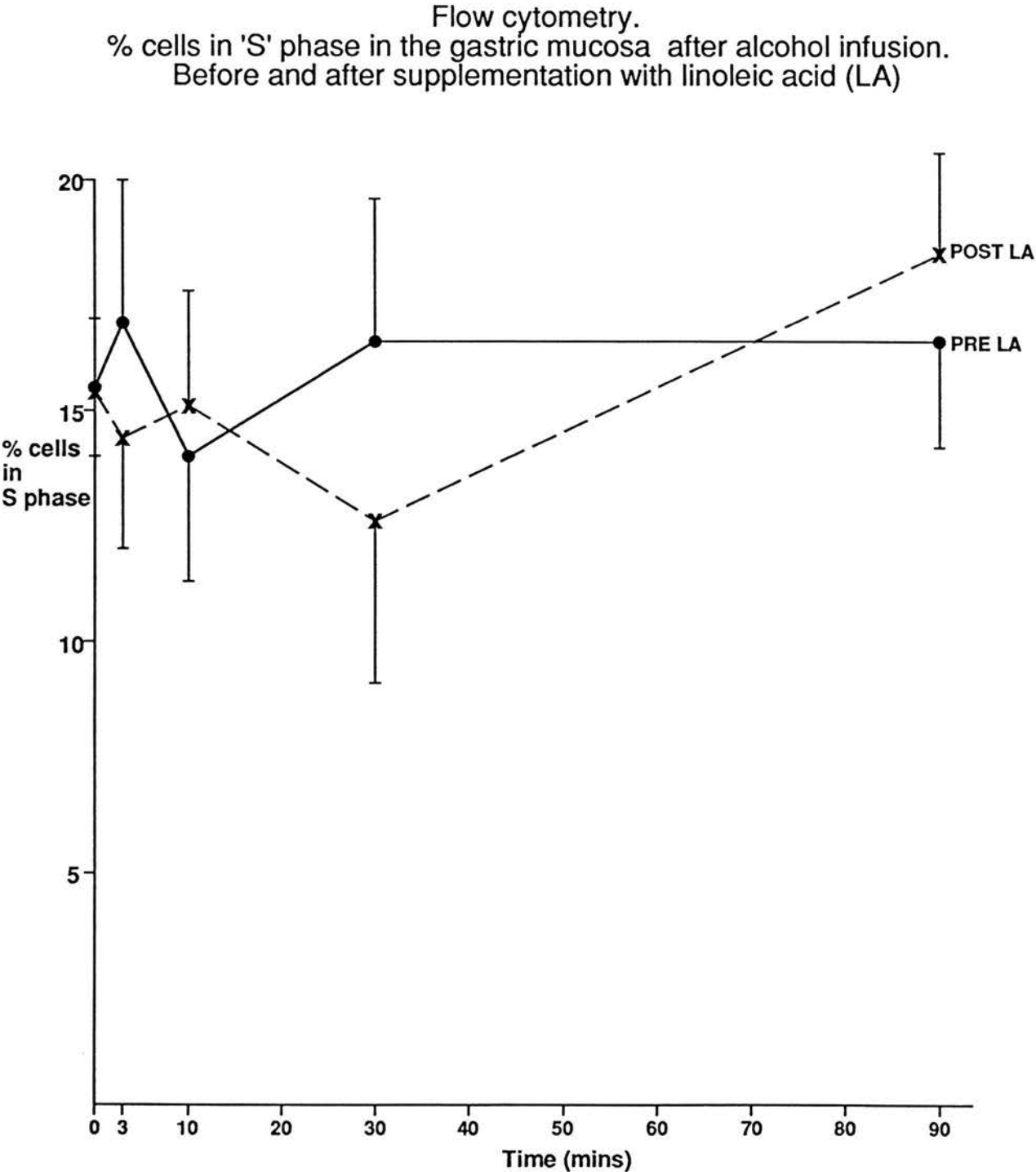


table 3:7

RESULTS FROM FLOW CYTOMETRY --- % OF CELLS
IN " S " PHASE OF CELL CYCLE

VOLUNTEER		TIME FROM ADMINISTRATION OF ALCOHOL (min)				
		0	3	10	30	90
1	pre LA	---	---	---	---	---
	post LA	---	---	---	---	---
2	pre LA	14.7	25.2	14.5	16.6	25.5
	post LA	9.3	5.7	9.4	4.1	14.4
3	pre LA	23.3	20.1	14.2	26.8	19.4
	post LA	14.4	21.8	12.4	17.5	19.4
4	pre LA	13.9	6.3	6.1	2.7	10.1
	post LA	20.4	19.2	16.7	14.9	12.7
5	pre LA	16.2	24.7	24.4	24.7	15.5
	post LA	18.6	12.9	19.0	15.1	20.1
6	pre LA	10.3	8.7	11.3	12.4	12.4
	post LA	15.1	12.2	18.1	12.1	26.0
mean	pre LA	15.6	16.9	14.0	16.6	16.6
	post LA	15.5	14.4	15.1	12.6	18.4

Fig 3:13



aspirin can cause bleeding throughout the gut and this can be reduced by orally administered prostaglandin analogues.

This study showed no reduction of blood loss when linoleic acid was given prior to aspirin. This was a little surprising as Cohen et al (1985) demonstrated a significant reduction in blood loss when aspirin was taken with Misoprostol (a prostaglandin E₁ analogue). Mean blood loss in the Misoprostol group was 3.75 ml per day compared to 6.05 ml per day in the placebo group. Similar reduction of aspirin-induced bleeding was also found with the PGE₂ analogue Enprostil. (Hawkey et al, 1986; Stiel et al, 1986). Enprostil reduced blood loss from a mean of 7.7 ml/10 minutes when aspirin alone was taken to 4.2 ml/10 minutes when Enprostil was taken prior to the aspirin.

The experiments described in Chapter 2 demonstrated a three-fold increase in PGE output following dietary supplementation with linoleic acid in normal subjects. It is not clear why this did not protect against aspirin. The findings are however supported by Pritchard P et al (1988) who found an increase in immunoreactive PGE₂ after dietary supplementation with evening primrose Oil (Efamol) which contains approximately 72% of linoleic acid (by weight). They also found that aspirin induced micro-bleeding was not affected by the increase in PGE₂. Mean blood loss from gastric washings after a five-day

course of aspirin was 10.7 ul/10 minutes and 9.4 ul/10 minutes when Efamol was taken prior to the aspirin.

One explanation for the lack of protection from aspirin relates to the dose of linoleic acid used in these experiments. The output of PGE occurring after linoleic acid is at least one order of magnitude less than that following ingestion of exogenous PGE or one of its analogues. It may well be that higher doses of linoleic acid might have led to higher PGE outputs and demonstrable protective effects. However, such high doses would cause unacceptable side effects, particularly diarrhoea.

A further reason for the lack of protection from aspirin associated blood loss is that aspirin inhibits cyclo-oxygenase enzymes, interrupting PGE synthesis. Consequently the anticipated increase in PGE secretion following linoleic acid might not have occurred. The design of this experiment precluded measurement of gastric PGE output and this possibility remains unproven. It was hoped however that the stimulating effect of linoleic acid upon prostaglandin synthesis would have competed with the aspirin effect, and reduce mucosal damage. Studies in rodents, reported by Konturek et al (1982) suggest that this was likely to be a vain hope. In a series of experiments this group showed that although "adaptive cytoprotection" protected against the damaging effects of ethanol and 25% NaCl, it had no

effect upon aspirin-associated gastric mucosal injury.

b) Alcohol induced injury

There are several possible explanations to account for the apparent failure of linoleic acid to protect against the damaging effects of ethanol on the gastric mucosa.

(i) The output of prostaglandins associated with linoleic acid may have been insufficient.

(ii) Biopsies were taken from the edge of the damaged area rather than the centre in order to prevent biopsy artefact.

(iii) Much of the metabolic evidence cited to support a role for essential fatty acids in peptic ulcer disease is derived from studies of experimentally induced ulcers in rats, in which either ethanol or aspirin were used to induce the gastric lesions (Hollander et al, 1982; Tarnawski et al, 1985). Both these agents inhibit Δ -6 desaturase, the rate limiting enzyme for the conversion of linoleic acid to arachidonic acid. Therefore the beneficial effects of the administration of these acids may be nullified by the presence of aspirin and alcohol (Huang et al, 1987; Wang and Reitz, 1983).

c) Linoleic acid and endogenous gastric prostaglandin secretion

Although it is widely accepted that orally administered (exogenous) prostaglandins and their analogues protect the stomach, the role of prostaglandins secreted by the stomach (endogenous prostaglandins) is less well understood. Endogenous prostaglandins have been studied in several ways -

(i) Inhibiting the formation of prostaglandins by NSAIDS. Although NSAIDS do inhibit cyclo-oxygenase enzymes and are associated with gastric mucosal injury, it does not necessarily follow that these effects are related.

(ii) Adaptive cytoprotection. This is the phenomenon whereby low doses of a damaging agent protect the gastric mucosa from higher doses of the same or another toxin. It is extremely indirect evidence for a role of endogenous prostaglandins since other factors may also be responsible.

The observation that linoleic acid increased gastric prostaglandin output whereas saturated fat did not, provides the basis of a much more "physiological" approach to the study of endogenously secreted gastric prostaglandins. This concept had previously been employed by others in experimental animals but no previous workers had studied man. Schepp et al (1988)

studied groups of rats whose diets were depleted or supplemented with linoleic acid. Linoleic acid-depleted diets were associated with markedly reduced PGE output and increased basal and maximal acid output. Damage induced by cold and restraint was increased compared to rats given normal diets. In contrast, rats with high linoleic acid diets exhibited increased PGE output, decreased acid secretion and decreased gastric mucosal injury. Tarnawski et al (1985) studied groups of rats whose diets were supplemented with Pluronic F68, oleic acid or two doses of linoleic acid (in Pluronic F68). 100% ethanol was infused into the stomach one hour after the last dose of fatty acid or solubiliser. In the two control groups histology and E-M revealed that more than 90% of the surface epithelium had been damaged and visual necrosis occupied 40-70 % of the surface area. In the two linoleic acid treated groups, histology and E-M revealed significantly reduced disruption of the surface epithelium (8%) and necrosis was visible over only 3-9% of the surface area. This protection only occurred if the linoleic acid was given intragastrically (and not when given intrajejunally) and gave rise to an increase in PGE output. In a similar series of experiments in rats the same group (Hollander D et al 1982) looked at the effect of arachidonic acid (AA) on mucosal protection against alcohol injury. They looked at the effects of alcohol on mucosal histology, E-M, mitotic index, ^3H -TdR, pH and electrolytes and mucosal

potential difference. They found that mucosal necrosis was a time-related process: those rats receiving arachidonic acid had less mucosal injury, less severe disruption of mitosis, the labelling index was higher, potential difference and pH recovered more quickly.

It is possible that endogenous prostaglandins, stimulated by linoleic acid, protect the human stomach but discernable benefits of linoleic acid were not found from the studies described in this chapter.

d) Comparison of flow cytometry with autoradiography

The mean values for the labelling index obtained from flow cytometry (15.6 %) mirrored those from autoradiography (17.7 %). Autoradiography is a reproducible and recognised technique whereas flow cytometry applied to gastric tissue is still being developed. Difficulties occur in preparing cell suspensions for flow cytometry and this can lead to problems of reproducibility. For these reasons autoradiography is regarded as the gold standard.

CHAPTER 4

DIETARY LINOLEIC ACID, GASTRIC PROSTAGLANDINS AND DUODENAL ULCER

1. INTRODUCTION

2. MATERIALS AND METHODS

subjects

adipose tissue sampling

gastric secretion

3. RESULTS

4. DISCUSSION

1. INTRODUCTION

As briefly discussed in chapter 1, there has been a decline in the incidence and virulence of peptic ulcer disease throughout the West (Mendeloff, 1974; Bonnevie, 1985) during this century. This is based upon a number of observations:-

i) There has been a gradual decline in hospital admissions over the last few decades (Elashoff and Grossman, 1980; Brown et al, 1976). This may be a reflection of decreasing incidence, a change in the severity of the disease or may be due to revised criteria for admission; peptic ulcer patients are now mainly treated as out-patients.

ii) There has been a decline in the incidence of perforated peptic ulceration (MacKay, 1966; Illingworth et al, 1944; Jamieson, 1955). This is a good index of virulence because all the patients are hospitalised. Perforation occurs in only a minority of ulcer patients - between 5 and 10% (Litton and Murdoch, 1963) and about 70% of perforations occur in patients without a preceeding history of peptic ulcer disease (Cassel, 1969).

iii) Prospective studies of the prevalence and epidemiology did not appear until 1950. These show considerable differences between countries and even within countries. In the United Kingdom the incidence of

duodenal ulcer increases as one travels northwards (Langman, 1979). A prospective study of first-time diagnosed cases of duodenal ulcer in males in New York city showed a significant fall over the years 1952 - 1963 (Pulvertaft, 1959; Pulvertaft, 1968). In Iceland Jonasson (1983) noted a less marked decline.

The factors associated with this falling incidence and virulence have been widely studied. These include powerful new ulcer healing drugs, a decline in smoking, enviromental factors including industrialisation, changes in work patterns, climate (Kurata and Haile, 1984; Friedman et al 1974) and changes in diet.

Ulcer healing drugs have been available since the late 1970's and their use has been associated with a marked fall in the number of operations performed for uncomplicated peptic ulcer. These drugs do not affect the development of an ulcer but they do induce a temporary remission. The falling incidence of peptic ulcer disease pre-dated the introduction of these new agents.

Cigarette smoking is associated with a decrease in ulcer healing (Sontag et al, 1984) and may be associated with the development of ulcers. The recent decline in smoking in the U.K. cannot be held responsible for this long-term trend.

People from social class V have a higher incidence of

peptic ulcer disease than those in social class I. The reasons are not known but may be related to environment, differences in smoking, diet and recreation.

While general improvement in nutrition could be relevant, it is conceivable that changes in specific dietary components could affect ulcer epidemiology. This thesis has shown that changes in dietary essential fatty acid consumption are associated with an alteration in the output of gastric prostaglandins and these have effects upon acid secretion and mucosal protection. Hollander and Tarnawski (1986) suggested that the observed 200% increase in linoleic acid consumption could account for the falling incidence and virulence of peptic ulcer. This chapter explores this hypothesis by comparing the fatty acid composition of adipose tissue obtained from duodenal ulcer and matched control patients.

Estimates of dietary intakes of linoleic acid are unreliable because of the paucity of data on the fatty acid composition of common foodstuffs and because of the inaccuracies inherent in the short term assessment of nutrient intakes in general (Roshanai and Saunders, 1984; Black, 1986). In contrast, adipose tissue levels of dietary linoleic acid accurately predict long-term intakes of dietary linoleic acid and have been used in many epidemiological and case control studies which examined the link between dietary intakes of linoleic

acid and coronary heart disease (Van Staveren et al, 1986; Fordyce et al, 1983; Wood et al, 1987).

The aims of this study were :-

- i) To compare adipose linoleic acid and fatty acid profiles in duodenal ulcer and control patients.
- ii) To determine whether the linoleic acid composition of adipose tissue bears any relationship to gastric acid and prostaglandin secretion.

2. METHODS

Subjects

Thirty five men with endoscopically proven chronic duodenal ulcers aged 21 - 79 years (mean 45) were studied. All had a long dyspeptic history but none had undergone surgery or was taking non-steroidal anti-inflammatory drugs or any ulcer-healing medications at the time of the study. Each ulcer patient was matched with a control male subject of similar age, social class, and smoking history. The control subjects comprised patients undergoing routine minor elective surgery (principally inguinal hernia repair and haemorrhoidectomy) and laboratory personnel. None of the controls had a history of dyspepsia or were taking any

drugs. Their ages ranged from 18 - 75 years (mean 46). Non-smoking was defined as never having smoked or having stopped for at least 6 months before the study; current smokers were matched for their daily cigarette consumption.

Adipose Tissue Sampling and Laboratory Analysis

Adipose tissue was sampled from the anterior wall: 2-3 mls of 1% lignocaine with adrenaline were first injected sub-cutaneously, and a 3 mm diameter Stieffel skin biopsy needle was then used to core out the skin and sub-epidermal fat. A small quantity of fat (no greater than 20 mg) was required for analysis. The fat was separated from the skin, rinsed with saline, dried on absorbant paper and stored in an Eppendorf container at -60°C.

Samples were assayed in a blind manner using the chromatographic method described by Wood et al (1987). Adipose tissue samples were thawed, rinsed in saline and the lipids were extracted into re-distilled heptane. The extract was washed with isopropanol/0.05% potassium hydroxide by volume, in order to remove non-esterified fatty acids and phospholipids. The neutral lipid extract was reduced to dryness under vacuum then dissolved in dry toluene. Fatty acid methyl-esters were prepared by direct transesterification (10 min at 50°C with 0.5 mol/l sodium methoxide in methanol). The fatty esters were

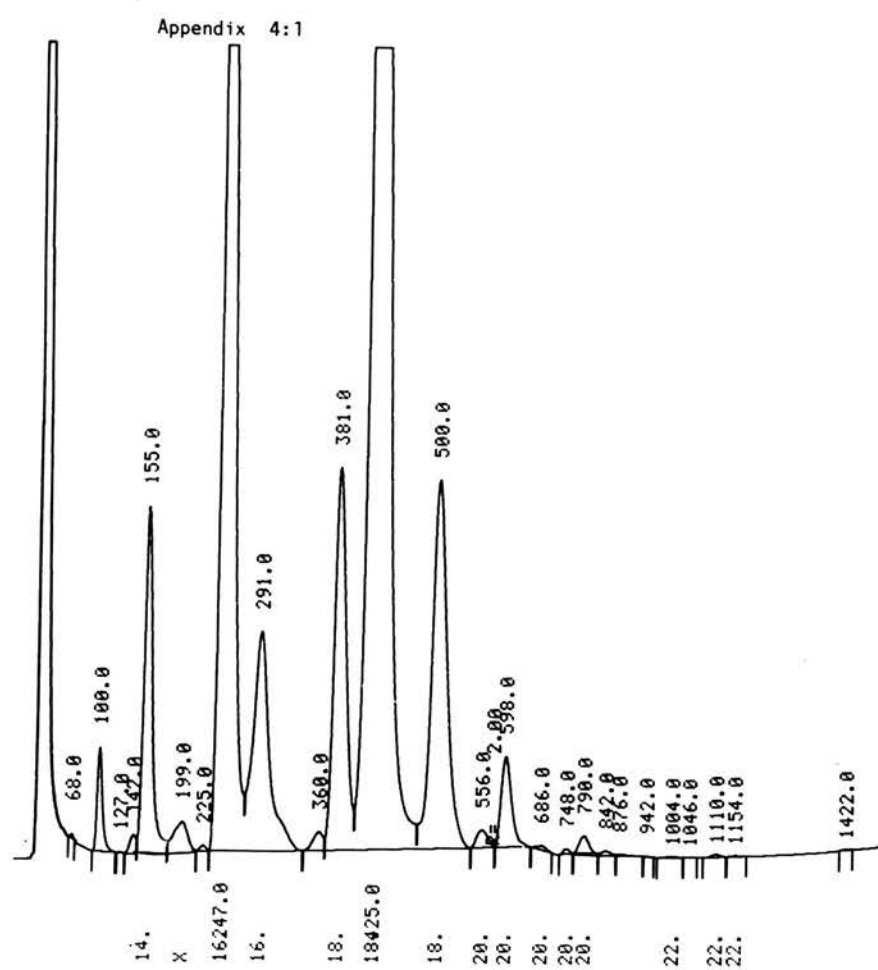
washed with acidified water and re-extracted into hexane: the hexane layer was evaporated under vacuum. The methyl esters were redissolved in 40 ul redistilled chloroform, ready for analysis on a Pye Chromatograph 204, fitted with a 1.5 m column, packed with GP 10%, SP-2330 on 100/120 mesh Chromosorb W AW (Supelco). The peaks were quantified with a flame ionisation detector and "Tri-vector III" integrator. The methyl-ester peaks in the chromatograms were identified by a combination of argentation thin layer chromatography (TLC) and comparison of retention times with those of authentic fatty-acid esters (Pufa 1 and 2 and NIH mixtures, Supelco). For complex peaks, GC/MS was used in a few random samples. A typical TLC printout is shown in Fig 4:1.

Gastric secretion studies

Thirteen ulcer subjects and seven controls underwent a pentagastrin secretion test. The method has been described in chapter 2. Gastric acid output was determined in 5 ml aliquots of gastric juice by titration to pH with 0.01 N NaOH using a N82 standard pH meter (Radiometer, Copenhagen). Gastric acid output was calculated from the product of concentration and volume. Basal output was derived from the juice collected before the pentagastrin and maximal acid output from the juice collected after the pentagastrin had been given. Prostaglandin output was measured from the gastric juice

Fig 4:1

Example of thin layer chromatography (TLC)
of fatty acid profile from adipose tissue



by the method previously described in Chapter 2. Output was calculated from the product of concentration and volume.

3. RESULTS

The % linoleic acid in the adipose tissue was significantly lower in the ulcer group when compared to the controls, and this was true for both smokers and non-smokers (Table 4:1). Wood et al (1987) also found that adipose fatty acid profile between smokers and non-smokers was different. There was no significant difference in the profiles of the other fatty acids between the 2 groups (tab 4:2).

Table 4:3 depicts the mean % linoleic acid in adipose tissue, prostaglandin and acid secretory status of the 7 control and 13 ulcer subjects who underwent the modified pentagastrin test. In the ulcer patients there was no relationship between % linoleic acid in adipose tissue and gastric PGE output ($r = 0.003$), PGEM output ($r = 0.04$), basal or stimulated acid secretion ($r = -0.02$ and 0.13 respectively).

In control subjects there was no relationship between % linoleic acid in adipose tissue and PGE and PGEM output ($r = 0.03$ and 0.04 respectively), but % linoleic acid in adipose tissue and basal acid output were significantly negatively correlated ($r = -0.74$, $p < 0.01$). The

table 4:1

LINOLEIC ACID COMPOSITION OF ADIPOSE
TISSUE (% composition W / W)

	OVERALL	SMOKERS	NON SMOKERS
duodenal ulcer linoleic acid	35 9.9 \pm 0.68	19 9.4 \pm 0.59	16 10.8 \pm 0.72
healthy controls linoleic acid	35 12.3 \pm 0.75	14 12.1 \pm 0.85	21 12.4 \pm 0.93

table 4:2

MEAN VALUES OF ADIPOSE TISSUE FATTY
ACIDS (% composition W / W)

FATTY ACIDS	HEALTHY CONTROLS	DUODENAL ULCER PATIENTS
palmitic acid (16:0)	22.7 \pm 0.55	21.9 \pm 0.34
palmitoleic acid (16:1)	6.1 \pm 0.28	7.9 \pm 0.28
stearic acid (18:0)	4.8 \pm 0.26	4.0 \pm 0.16
Oleic acid (18:1)	47.1 \pm 0.48	47.7 \pm 0.78
LINOLEIC ACID(18:2)	12.3 \pm 0.75	9.9 \pm 0.68

Tab 4:3

Comparison of mean values of % linoleic acid in adipose tissue with mean values of PGE output , basal acid output (BAO) , and maximal acid output (MAO) in healthy controls and duodenal ulcer patients.

	% linoleic acid in adipose tissue	mean PGE ng/hr	BAO mmol/hr	MAO mmol/hr
controls n = 7	12.8 (1.1)	614 (101)	4.5 (1.3)	29.4 (4.5)
DU patients n = 13	11.0 (1.3)	89 (14)	3.2 (0.5)	30.8 (3.7)

relationship between % linoleic acid in adipose tissue and basal acid output is shown in Fig 4:2. Adipose % linoleic acid and maximal acid output were also inversely related ($r = -0.58$, $p < 0.05$): this is illustrated in Fig 4:3.

DISCUSSION

The study has shown that mean dietary linoleic acid intake was lower in male duodenal ulcer patients than in control subjects of similar age, smoking habits and social class. These findings are consistent with the hypothesis that dietary essential fatty acids influence the natural history of duodenal ulcer.

Although there was no relationship between % linoleic acid in adipose tissue and gastric prostaglandin output, it is likely that the effect of linoleic acid upon ulcers is mediated through prostaglandins. Gastric prostaglandin output is a crude reflection of intracellular events and it is possible that subtle changes in prostaglandin metabolism result in changes of mucosal protection and acid secretion. Of interest is the significant negative relationship observed between % linoleic acid in adipose tissue and acid secretion which was only seen in control subjects. In Chapter 2 it was shown that in normal subjects dietary linoleic acid increased gastric prostaglandin secretion and suppressed

Fig 4:2 RELATIONSHIP OF ADIPOSE TISSUE % LINOLEIC ACID AND BASAL ACID OUTPUT (BAO) IN HEALTHY CONTROLS

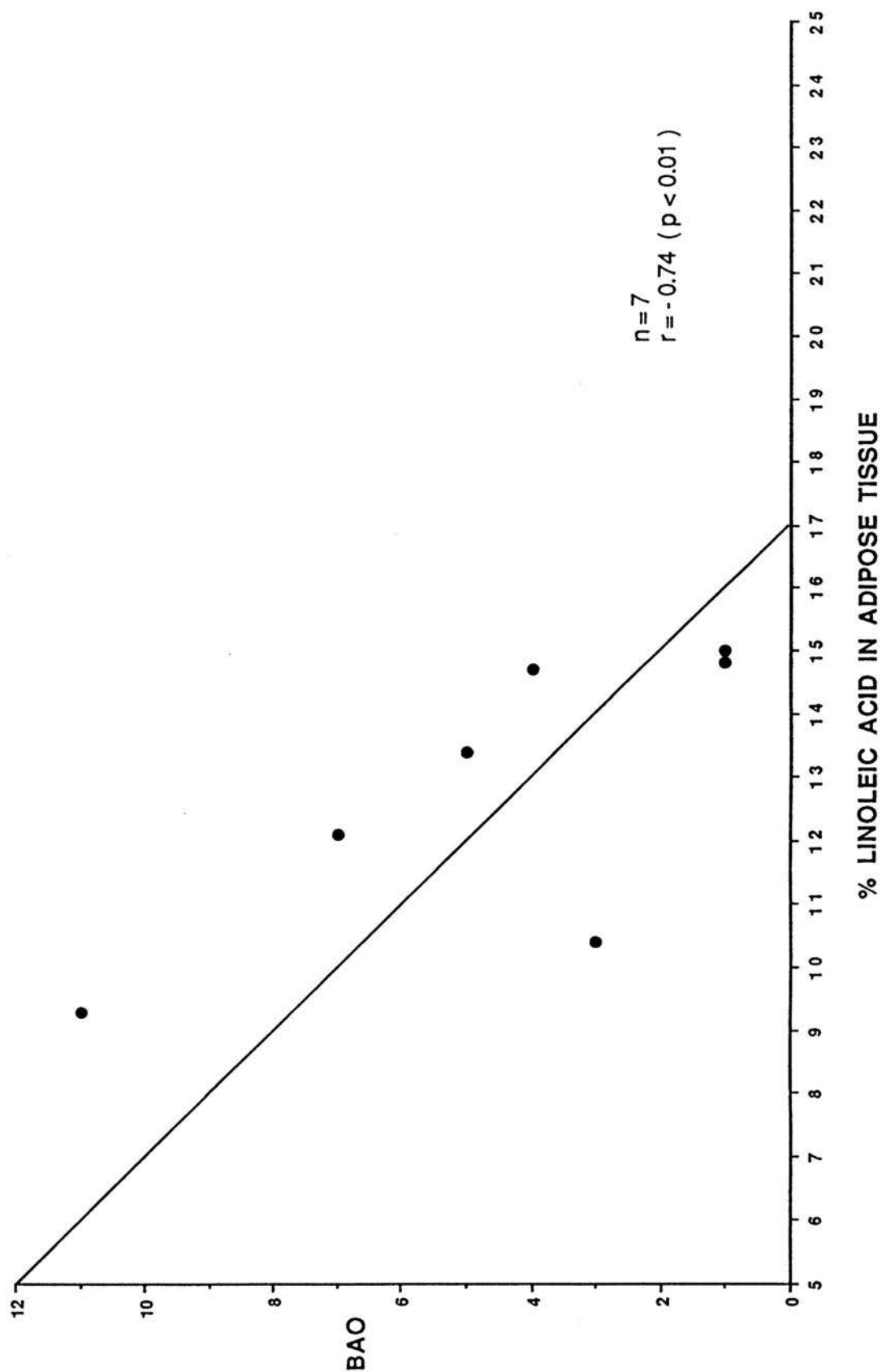
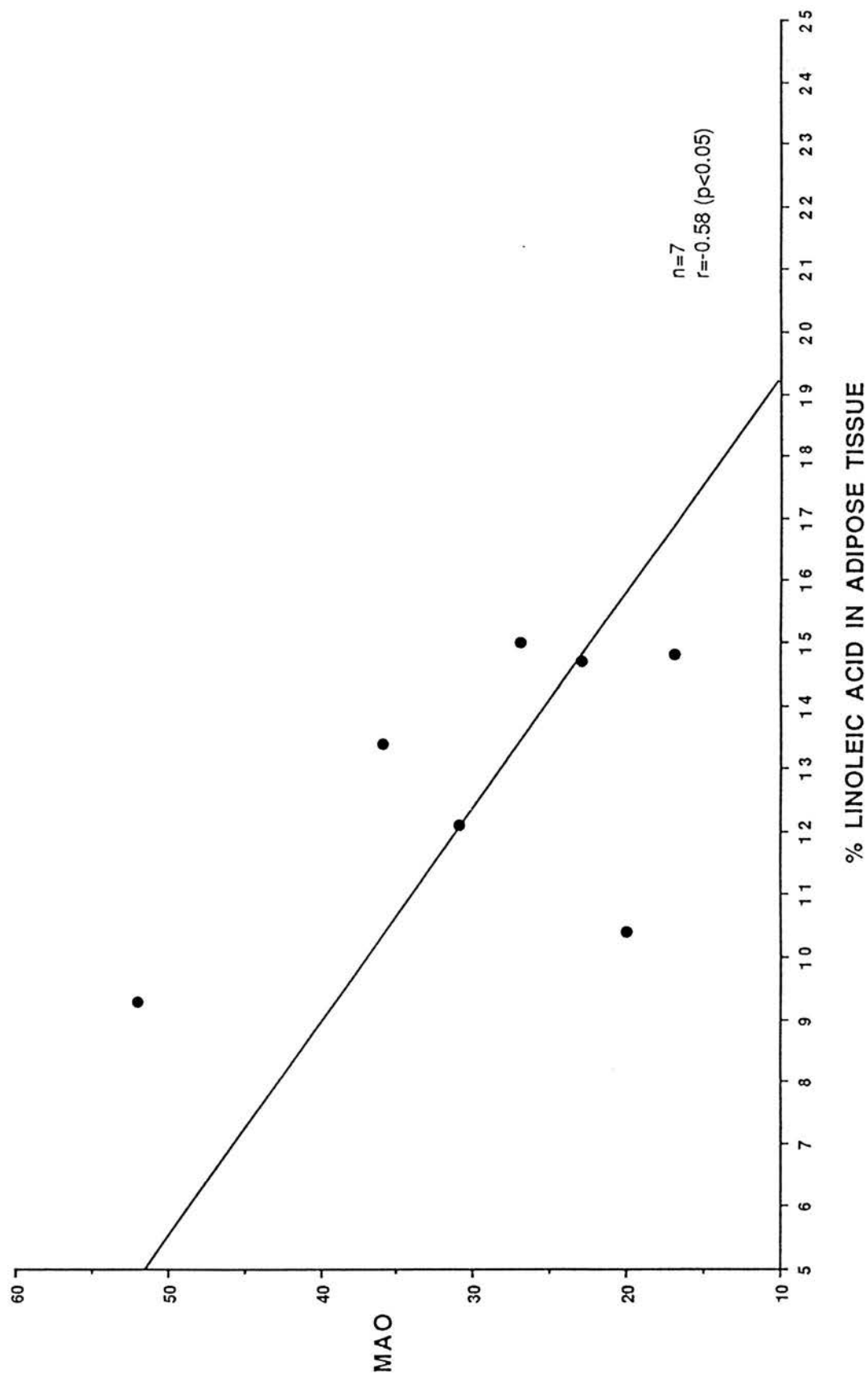


Fig 4:3 RELATIONSHIP OF ADIPOSE TISSUE % LINOLEIC ACID AND MAXIMAL ACID OUTPUT (MAO) IN HEALTHY CONTROLS



acid output, but this did not occur in patients with duodenal ulcer. It may therefore be that normal subjects can protect their gastric mucosa by consuming essential fatty acids. This does not occur in duodenal ulcer because these patients do not respond to dietary linoleic acid by increasing gastric prostaglandin secretion. From this study it is not clear whether the lower % linoleic acid found in the adipose tissue of duodenal ulcer subjects may be a factor in the aetiology of their duodenal ulcer disease, or whether it is due to a change in their diet as a result of their dyspepsia. It is unlikely to be the latter as many medicines and remedies which relieve dyspeptic symptoms contain essential fatty acids.

CHAPTER 5

1. **SUMMARY**
2. **CONCLUSION**
3. **WHAT IS NEW?**
4. **THE WAY AHEAD**

1. SUMMARY

During the course of this study several interesting facts have emerged. Linoleic acid administered orally in modest doses gave rise to a significant increase in the output of PGE and PGEM in normal healthy controls. This is a specific feature of linoleic acid and not merely a non-specific effect of fatty acids, because controls given a saturated fatty acid stearic acid (which is not a prostaglandin precursor) had no effect on PGE and PGEM output. This dietary stimulation of endogenous PGE is an immediate effect which wanes rapidly over 1 hour. In patients with proven duodenal ulcer disease gastric PGE output was much lower than that found in controls and did not increase in response to linoleic acid. More of the prostaglandin was found as its metabolite 13,14-dihydro 15-keto PGE₂. This suggested that there is a difference in the handling of dietary prostaglandin precursors in duodenal ulcer subjects and that prostaglandin catabolism is increased.

This difference in PGE output was mirrored by the change in acid output after linoleic acid administration. In the controls, but not in the ulcer patients, gastric acid output was significantly decreased by dietary linoleic acid. The reduced acid secretion in the control group was probably secondary to the significant rise in gastric PGE secretion.

A difference was also found in the concentration of serum gastrin after linoleic acid. In the normal subjects, there was a significant increase in serum gastrin concentration after linoleic acid. This did not occur in the control group taking stearic acid nor in the duodenal ulcer group. It is difficult to explain this difference. A rise in gastrin is found in people taking acid reducing drugs (e.g. H₂ blockers), however in this study acid suppression was fairly modest and therefore unlikely to be the reason for the increase.

Orally administered (exogenous) prostaglandins have been shown to protect the gastric mucosa from many types of injurious agents, however the importance of locally secreted prostaglandins is less well understood. Most of the evidence for a role of endogenous prostaglandins is indirect and comes from the phenomenon of "adaptive cytoprotection".

In this study an alternative approach to the investigation of the protective effects of endogenous PGE was adopted - namely the stimulation of endogenous PGE by linoleic acid. Three approaches were used to study the protective effect of endogenous PGE - mucus output, aspirin-induced injury and alcohol-induced injury.

Dietary linoleic acid increased mucus output in controls but not in duodenal ulcer subjects.

Exogenous prostaglandins and their analogues are known to

protect the stomach from injury by both aspirin and alcohol but in this study linoleic acid had no such effect. This may be a dose related phenomenon, the levels of endogenous PGE found in this study were much lower than the doses of exogenous PGE administered in other studies. It could also be that both aspirin and alcohol prevented the anticipated rise in gastric prostaglandin secretion following linoleic acid.

At a cellular level no increase in protection after stimulation of endogenous PGE was found - in contrast with the effect of exogenous PGE. This again, could be a dose-related phenomenon.

Overall it was found that the induction of PGE had no significant beneficial effect in the models of injury that were studied.

Adipose tissue linoleic acid levels, which reflect chronic dietary habits, were significantly lower in duodenal ulcer patients than in matched control subjects. This difference occurred in smokers and in non-smokers. In the healthy controls, but not in the ulcer patients, there was a significant negative correlation between gastric acid secretion and the % linoleic acid in adipose tissue. No relationship was found between % linoleic acid in adipose tissue and gastric prostaglandin secretion in either group.

2. CONCLUSION

The thesis set out to examine the hypothesis of Hollander and Tarnawski that the natural history of duodenal ulcer could have been influenced by changes in dietary essential fatty acid intake. The evidence derived from these studies partly supports this contention and provides further insight into prostaglandin metabolism in duodenal ulcer disease.

While gastric prostaglandin secretion was not increased in duodenal ulcer subjects receiving linoleic acid, the observation that normal subjects did exhibit this response, coupled with a fall in gastric acid secretion in these individuals, suggests that linoleic acid can maintain a benign gastro-duodenal environment in normal subjects. In ulcer patients dietary linoleic acid had no effect on gastric secretion and there was no advantage in taking the fatty acid. This is reflected in the differences in adipose fatty acid profiles in the control and ulcer groups. Duodenal ulcer patients take less linoleic acid in their diet. This may be of aetiological significance in peptic ulcer disease.

The reasons for the different handling of linoleic acid between healthy controls and ulcer patients cannot be answered by the data presented in this thesis. The low secretion of PGE and the relatively high levels of PGEM in the ulcer group suggest increased PGE catabolism

rather than a failure of PGE synthesis. On the other hand the lack of increase in PGE output following linoleic acid suggests an additional defect in the incorporation of linoleic acid into the prostaglandin cascade. Only further studies involving incubation of biopsies could clarify the situation.

The role of endogenous PGE in protection of the gastric mucosa from injurious agents is inconclusive. While exogenous PGE appears to be protective, endogenous PGE in this study showed few beneficial properties.

3. WHAT IS NEW ?

This research has highlighted several new aspects to duodenal ulcer disease.

- It has demonstrated a different way of studying endogenous prostaglandins.
- It has demonstrated a method of stimulating endogenous prostaglandins using linoleic acid in its free fatty acid form.
- It has highlighted differences in gastric physiology between normal controls and patients with duodenal ulcer. The two groups respond differently to linoleic acid in terms of their PGE output, gastric acid secretion, mucus output

and fasting serum gastrin concentration.

- It has helped to clarify some of the conflicting results in the literature regarding prostaglandin output in health and in duodenal ulcer disease.
- It has contrasted the dramatic effects of exogenous prostaglandins as reported in the literature with the modest effects of endogenous prostaglandins.
- It has demonstrated that duodenal ulcer patients take less linoleic acid in their diet than healthy controls. This may be another aetiological factor in peptic ulcer disease.
- It has utilised new techniques in radioimmunoassay which improve its sensitivity and specificity - namely using methyloximated derivatives.
- It has contrasted several complementary methods of assessing mucosal injury - empirically by measuring aspirin-induced blood loss, and at a cellular level by autoradiography and the relatively new technique of flow cytometry.

4. THE WAY AHEAD

It would be useful to pursue this avenue of research further.

- i) To investigate whether much larger doses of linoleic acid could reduce aspirin-induced bleeding and alcohol-induced injury.
- ii) To perform more extensive dose-related studies into linoleic acid and PGE output.
- iii) To investigate further the differences between the control group and the ulcer patients - to demonstrate why there should be a block in utilising linoleic acid in the ulcer patients and at what level this occurs; to find out why there should be increased metabolism of PGE in the ulcer patients.
- iv) To perform a larger epidemiological study on the dietary intake in the two groups.

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Dietary Linoleic Acid, Gastric Acid, and Prostaglandin Secretion

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Basal and pentagastrin-stimulated gastric acid secretion, fasting serum gastrin concentrations, and the gastric output of prostaglandin E and its major metabolite 13,14-dihydro 15-keto prostaglandin E₂ were measured in 9 normal subjects before and after 14–20 days of dietary supplementation with linoleic acid. Mean maximal gastric acid output fell from 36.0 ± 3.3 (SEM) to 30.1 ± 2.9 mmol/h ($p < 0.05$), although mean basal acid output was not significantly affected (8.3 ± 2.1 and 7.2 ± 1.7 mmol/h, respectively). Mean fasting serum gastrin concentrations increased from 19.2 ± 3.1 to 30.9 ± 3.8 ng/L ($p < 0.01$) after linoleic acid, probably because of acid suppression. The mean output of prostaglandin E increased from 498 ± 110 to 1254 ± 465 ng/h ($p < 0.05$); that of its metabolite increased from 165 ± 18 to 1168 ± 645 ng/h ($p < 0.01$). These findings show that in normal subjects essential fatty acid weakly inhibits gastric acid secretion, but considerably increases gastric prostaglandin output.

In the Western hemisphere the incidence and virulence of peptic ulcer have decreased during the past four decades (1), whereas the average per capita consumption of polyunsaturated fatty acids has increased by >300% (2). Hollander and Tarnawski (3) have suggested that these two observations are related. Feeding polyunsaturated essential fatty acids (linoleic and arachidonic acids) to experimental animals leads to massive increases in prostaglandin concentrations in gastric juice (4,5) and these prevent experimental mucosal injury (5). Prostaglandins and prostaglandin analogues also profoundly affect human gastric function. In pharmacologic doses they suppress acid secretion (6), stimulate secretion of mucus and bicarbonate (7,8), and protect the gastric mucosa against the harmful effects of salicylates (9), bile acids (10), and ethanol (11). Their effects on gastrin release are controversial; they have

been variously reported to decrease (12) or have no effect on (13) serum gastrin concentrations. Increased intake of polyunsaturated fatty acids could therefore lead to lowered acid secretion and increased gastric mucosal protection. In this study the effects of oral linoleic acid on the gastric mucosal metabolism of prostaglandins, gastric acid secretion, and plasma concentrations of gastrin were measured in normal subjects. Linoleic rather than arachidonic acid was chosen because of its greater chemical stability and because linoleic acid is the major dietary polyunsaturated fatty acid. Free linoleic acid rather than the naturally occurring triglyceride was used as this simplifies its incorporation into the biosynthetic pathway of prostaglandins.

Materials and Methods

Materials

Linoleic acid was prepared as a micellar solution in nonionic detergent (Pluronic F68). Experiments in animals (4) have shown Pluronic F68 to be completely nontoxic and to have no effect on prostaglandin metabolism. Gelatin-coated capsules each containing 500 mg of linoleic acid and 320 mg of Pluronic F68 (Au-C003) were prepared by International Pharmaceuticals Inc., Costa Mesa, Calif. Capsules rapidly dispersed in both water and gastric acid at room and body temperature.

Subjects

Nine healthy, nonsmoking, white male volunteers (age range 20–41 yr) were studied. None had dyspepsia or gastroduodenal disease, abused alcohol, or consumed drugs of any description. All were taking a normal western diet before the study and were instructed not to change it during the study period.

Design of the Study

After an overnight fast venous blood was taken from each subject for measurement of hemoglobin concentration, white cell count, platelet count, erythrocyte sedimentation rate, and serum urea, creatinine, electrolytes, bilirubin, aspartate transaminase, glucose, thyroxine, and gastrin concentrations. Upper gastrointestinal endoscopy was performed using an Olympus XQ10 forward-viewing endoscope (Olympus Corp. of America, New Hyde Park, N.Y.). Lignocaine spray was used to anesthetize the pharynx but intravenous sedation was not given. Two biopsy specimens, each measuring $\sim 2-4 \times 2$ mm, were taken from the gastric antrum and the first part of the duodenum and processed for light and electron microscopy. Specimens for light microscopy were embedded in paraffin and stained using hematoxylin and eosin. Sections for electron microscopy were embedded in plastic, then impregnated with uranyl acetate and lead citrate. Three grids were examined in a JEOL CX2 transmission electron microscope (JEOL USA, Peabody, Mass.) using an operating voltage of 60 kV. After endoscopy a nasogastric tube was swallowed and the gastric contents were aspirated under continuous suction at 15-min intervals for 75 min. Pentagastrin (6 $\mu\text{g/kg}$) was then injected subcutaneously and four further 15-min aspirates were collected. The subjects were subsequently randomized to receive three capsules (1.5 g of linoleic acid and 960 mg of Pluronic F68) or six capsules (3 g of linoleic acid and 1920 mg of Pluronic F68) taken in equal divided doses at eight hourly intervals. They were interviewed after 2-4 days to assess compliance and adverse effects and then studied again 14-19 days after starting linoleic acid. Exactly 1 h after the last dose of linoleic acid, blood was taken for hematology and biochemistry studies and serum gastrin concentration measurement. Upper gastrointestinal endoscopy with biopsy and a pentagastrin-stimulated acid study exactly as described above were again performed.

Assays

Serum gastrin concentrations were measured by radioimmunoassay (Department of Biochemistry, Glasgow Royal Infirmary). Rabbit antigastrin serum was used. The sensitivity was 15.24 ng/L (normal range 30-120 ng/L).

Gastric acid concentrations were determined in 5-ml aliquots of gastric juice by titration to pH 7 with 0.1 M NaOH using a PHM 82 standard pH meter (Radiometer, Copenhagen, Denmark). Gastric acid outputs were calculated from the product of concentration and volume. Basal acid output was derived from the juice collected before pentagastrin and maximal acid output from the sum of the four aliquots taken after pentagastrin. Prostaglandin concentrations were measured in samples of gastric juice that had been stored at -20°C . After aspiration each sample was immediately adjusted to pH 7 using 0.1 M NaOH to minimize conversion of prostaglandin E (PGE) to prostaglandin A (PGA) and then frozen. Prostaglandin E and its major metabolite 13,14-dihydro 15-keto PGE₂ were measured exactly as described by Kelly et al. (14). Briefly, antisera to conjugated PGE₂ and 13,14-dihydro 15-keto PGE₂ were raised in New Zealand white rabbits. Radioimmunoassay of methyl oximated samples was done using polyethylene glycol precipitation of bound label to which bovine γ -globulin had been added. Specificity had previously been determined by gas chromatography and mass spectrometry. There was 53% cross-reactivity between PGE₁ and PGE₂ and 31% between PGE₃ and PGE₂, but no reactivity with PGA or 6-oxo PGF_{1 α} . Interassay and intraassay precision of 13,14-dihydro 15-keto PGE₂ were 9.4 and 4.9-7.6, respectively (relative standard deviations). Those for PGE were 14.8 and 13.5-26.4, respectively. Prostaglandin outputs were calculated from the products of concentration and volume.

Ethical permission was obtained from the Lothian Ethics of Medical Research Sub-Committee for Medicine and Clinical Oncology.

Statistical Methods

The Wilcoxon rank sum test for paired data and Student's paired *t*-test were used where appropriate.

Results

Endoscopic appearances of the stomach and duodenum, light and electron microscopic findings, and routine hematology and biochemistry studies were normal and unchanged throughout the study

Table 1. Mean (\pm SEM) Gastric Acid Secretion, Fasting Serum Gastrin Concentrations, and Total Gastric Prostaglandin Outputs Before and After Dietary Linoleic Acid

Variable	1.5 g LA (n = 5)		3.0 g LA (n = 4)		All subjects (n = 9)	
	Before	After	Before	After	Before	After
Basal acid output (mmol/h)	9.94 \pm 3.0	8.52 \pm 2.4	6.18 \pm 2.9	5.54 \pm 2.4	8.3 \pm 2.1	7.2 \pm 1.7
Maximal acid output (mmol/h)	36.67 \pm 2.88	31.09 \pm 3.58	35.24 \pm 7.09	28.88 \pm 5.4	36.0 \pm 3.3	30.9 \pm 2.9
Fasting serum gastrin concentration (ng/L)	22.4 \pm 4.8	33.4 \pm 4.6	15.25 \pm 3.7	27.75 \pm 7.12	19.2 \pm 3.2	30.9 \pm 3.8 ^a
PGE output (ng/h)	452 \pm 77	744 \pm 127	554 \pm 246	1891 \pm 1014	498 \pm 110	1254 \pm 465 ^b
PGEM output (ng/h)	154 \pm 20	274 \pm 84	180 \pm 34	2287 \pm 1318	165 \pm 18	1168 \pm 645 ^a

LA, linoleic acid; PGE, prostaglandin E; PGEM, 13,14-dihydro 15-keto prostaglandin E₂. ^a *p* < 0.01, Student's *t*-test. ^b *p* < 0.05, Student's *t*-test.

period in all subjects. Five subjects were randomized to 1.5 g of linoleic acid; 4 other subjects received 3 g daily. Four subjects developed mild diarrhea during the study, which ceased on discontinuing linoleic acid. One subject experienced an exacerbation of pain from a chronic musculoskeletal injury. All subjects were nevertheless fully compliant. The effects of oral linoleic acid on gastric acid secretion, fasting serum gastrin concentrations, and gastric prostaglandin output are summarized in Table 1. Although basal acid secretion tended to decrease after treatment with linoleic acid, this trend just failed to achieve statistical significance ($p < 0.06$). There was however a small but significant fall in mean pentagastrin-stimulated gastric acid output after linoleic acid. Inhibition of acid secretion tended to be greater in those subjects who received 3 g rather than 1.5 g per day, although the small numbers preclude a statistical analysis. The results of mean gastric acid secretion before and after linoleic acid ingestion are depicted in Figure 1. Fasting serum gastrin concentrations increased after linoleic acid ingestion in all but 1 subject (Figure 2). There was no obvious relationship between serum gastrin concentrations and the daily dose of linoleic acid, nor with basal or pentagastrin-stimulated acid secretion. Gastric PGE output increased markedly after linoleic acid (Figure 3) as did the output of its

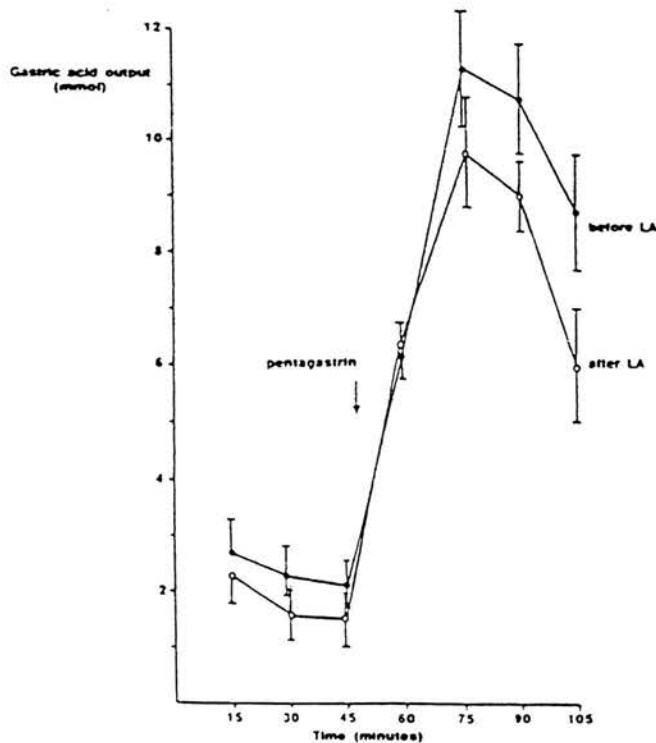


Figure 1. Mean (\pm SEM) basal and pentagastrin-stimulated gastric acid output before and after linoleic acid (LA).

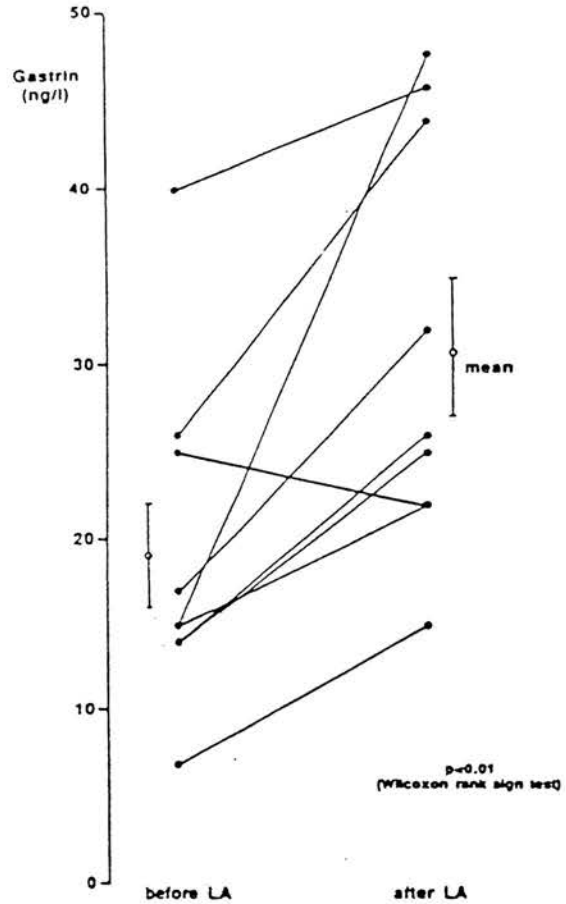


Figure 2. Fasting serum gastrin concentrations before and after linoleic acid (LA). Subjects received 1.5 or 3.0 g of linoleic acid daily.

major metabolite 13,14-dihydro 15-keto PGE₂ (Figure 4). The highest values were present in the first gastric aspirates, but outputs were also significantly higher throughout the period of gastric aspiration. Concentration rather than volume changes were responsible for this increase.

Discussion

This study has shown that in normal individuals dietary supplementation with relatively modest amounts of linoleic acid results in a small, but significant, fall in mean pentagastrin-stimulated gastric acid secretion and increased mean fasting serum gastrin concentrations. We suggest that these effects are the result of increased gastric prostaglandin synthesis.

The modest fall in gastric acid secretion suggests that linoleic acid will not be a potent duodenal ulcer healing agent. It is nevertheless interesting that olive and linseed oils, both rich in linoleic acid, were once traditional remedies for dyspepsia. Acid secretion

tends to be high in patients with duodenal ulcer (15), and the effect of linoleic acid on acid secretion observed in this study could therefore account for the observed inverse relationship between the incidence of peptic ulcer and per capita consumption of polyunsaturated fat. There is evidence that in addition to the effect on gastric acid secretion, prostaglandins are involved in the prevention of gastroduodenal ulcers and healing of ulcers by factors enhancing mucosal defense rather than ones that are independent of acid suppression (16). Stimulation by linoleic acid of endogenous gastroduodenal prostaglandin formation may therefore also affect the natural history of peptic ulcer by enhancing such "mucosal protective" factors.

The effects of prostaglandins and prostaglandin analogues on serum gastrin concentration are controversial. Tytgat and Huibiegtse (13) reported that mean serum gastrin concentrations in duodenal ulcer patients were unchanged after treatment with 15 (R)-15-methyl PGE₂, whereas Mahachai et al. (12) observed a significant fall after 1 wk of the PGE₂ analogue enprostil. It has been suggested that prostaglandins may suppress gastric acid secretion by decreasing gastrin release (12). It is likely that serum gastrin concentrations increased in our experiment as the result of gastric acid suppression. Similar

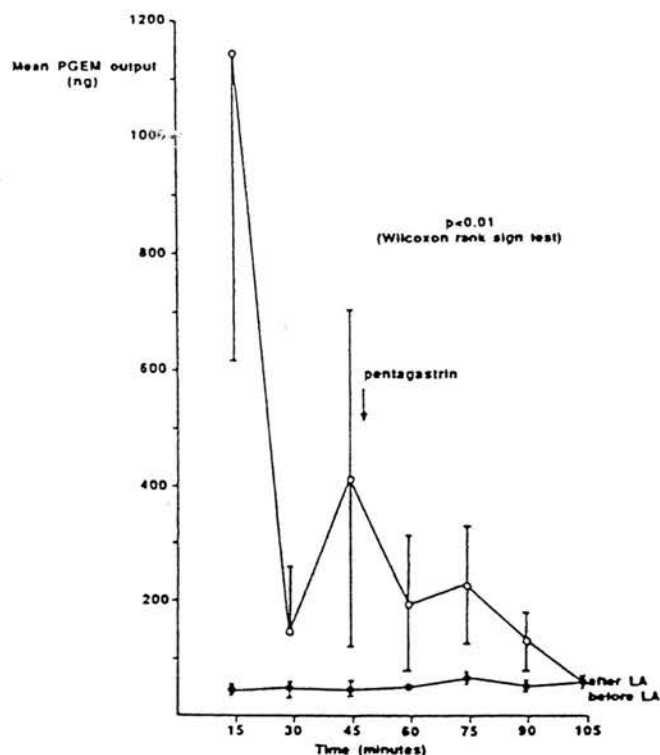


Figure 4. Mean gastric 13,14-dihydro 15-keto PGE₂ (PGEM) output before and after linoleic acid (LA).

effects occur with other agents that suppress gastric acid secretion (17,18).

The output of PGE reported in this study before dietary linoleic acid supplementation is similar to that found in a group of normal subjects by Cheung et al. (19). These workers collected samples by nasogastric tube without prior endoscopy or gastric mucosal biopsy. It is therefore unlikely that the trauma of endoscopy and mucosal biopsy significantly affected prostaglandin output in our study. Each subject acted as his own control and changes in PGE, 13,14-dihydro 15-keto PGE₂, and acid output and the increase in serum gastrin concentrations after linoleic acid cannot therefore be explained by this theoretical artifact. The increase in gastric prostaglandin output observed in this study is several orders less than that observed in rodents (4,5). It is possible that the peak prostaglandin concentrations occur almost immediately after linoleic acid. Our observation that early prostaglandin concentrations were highest supports this suggestion, although an altered response to the trauma of endoscopy, biopsy, and nasogastric intubation is an alternative possibility. We chose to start aspirating the stomach contents 1 h after the subjects swallowed the last capsules because earlier sampling would have removed significant amounts of linoleic acid. We cannot tell whether the effects of linoleic

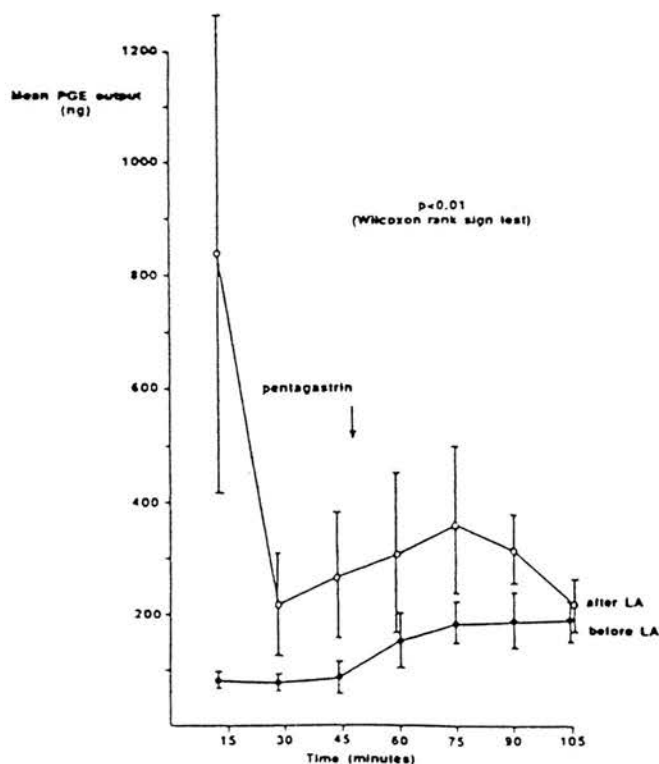


Figure 3. Mean (\pm SEM) gastric prostaglandin E output before and after linoleic acid (LA).

acid on gastric secretion and serum gastrin concentrations are due to chronic administration or would follow a single dose of linoleic acid. We speculate that dietary linoleic acid passively diffuses down its concentration gradient into gastric mucosal cells and is then incorporated into the prostaglandin cascade, leading to an outpouring of PGE into gastric juice. The early peak in PGE output in experimental animals (4) and humans supports this and suggests that the effects of linoleic acid are acute rather than due to chronic administration. Whether the approximately threefold increase in prostaglandin output could be physiologically, pharmacologically, or therapeutically important is unknown. We have demonstrated small, but significant, changes in acid and gastrin concentrations and others have suggested that prostaglandin analogues heal ulcers by so-called cytoprotective mechanisms in addition to their acid-suppressing effect (16). Although our observations are compatible with the hypothesis of Hollander and Tarawski (3), this study is obviously some way from proving an association between peptic ulcer and polyunsaturated fat ingestion. A study of peptic ulcer patients is clearly necessary, particularly as gastroduodenal prostaglandin metabolism may be abnormal in this disease (19,20).

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